MAY-JUNE 1954

The Journal of Infectious Diseases

Issued by the John Phillips Memorial Committee of the Henrietta Hospital
Under the Editorship of LUDWIG HEKTOEN and EDWIN O. JORDAN

WILLIAM BRADLEY COLEY, Editor-in-Chief

Advisory Editors

HOWARD G. MA... DACHE, M.D. GORDON R. W. HARRISON, M.D.
LEWIS L. LACHE... M.D. LOUIS KARL F. MEYER, M.D.
MOELLER, E. G. ... ROBERT RUSTIGIAN, DAVID W. ...

PUBLISHED BY THE JOHN D. ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
OF THE UNIVERSITY OF CHICAGO

THE UNIVERSITY OF CHICAGO PRESS
CHICAGO 37, ILLINOIS, U.S.A.

The Journal of Infectious Diseases

The Journal of Infectious Diseases is devoted to the publication of original investigations dealing with the general phenomena, causation and prevention of infectious diseases. It is the aim of the journal to occupy a special field and to include only such contributions as bear with reasonable directness on the topics indicated in the title. The biology and chemistry of the various pathogenic organisms, the physiology and anatomy of the morbid processes that they initiate, and the hygienic and sanitary problems to which they give rise are considered to be especially within the scope of the undertaking.

This journal was established in connection with the John Rockefeller McCormick Memorial Institute for Infectious Diseases, by the munificence of the late Mrs. Edith Rockefeller McCormick and the late Mr. Harold F. McCormick. Since 1941 it has been published in connection with the John Rockefeller McCormick Memorial Fund of the University of Chicago.

The Journal of Infectious Diseases is published bi-monthly, at The University of Chicago Press, 5750 Ellis Avenue, Chicago, Illinois. The subscription price is $7.50 per year; $3.50 a volume; the price of single copies of current volume is $2.00. ¶ Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Puerto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Haiti, Uruguay, Paraguay, Hawaiian Islands, Balearic Islands, Spain and Venezuela. ¶ Postage is charged extra as follows: for Canada and Newfoundland, 40 cents on annual subscriptions (total $7.40); on single copies 10 cents (total $2.10); for all other countries in the Postal Union, 75 cents on annual subscriptions (total $7.75); on single copies 15 cents (total $2.15). ¶ Patrons are requested to make all remittances payable to the University Press in postal or express money orders or bank drafts.

The following is an authorized agent:

For the British Empire, except North America, and Australasia: The Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, England. Prices of yearly subscriptions and on single copies may be had on application.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, 5750 Ellis Avenue, Chicago 37, Illinois.

Communications for the editors and manuscripts should be addressed to Mrs. Dorothy W. Green, Assistant to the Editor, The Journal of Infectious Diseases, 5734 Ellis Avenue, Chicago 37, Illinois.

Entered as second-class matter August 1, 1911, at the postoffice at Chicago, Illinois, under the Act of March 3, 1879.

Acceptance for mailing at special rate of postage provided for in United States Postal Act of October 3, 1917, Section 1103, amended February 28, 1925, authorized July 3, 1918.

Notice to Subscribers: If you change your address, please notify us and your local postmaster immediately. The Post Office does not forward second-class mail.

[PRINTED IN U.S.A.]

J. INFECT. DIS. VOL. 94, NO. 3

THE USE OF STREPTOMYCIN-RESISTANT AND NONRESISTANT STRAINS FOR THE DETERMINATION OF THE IMMUNIZING EFFECT OF LIVING *BRUCELLA ABORTUS* VACCINES IN WHITE MICE

A. L. OLITZKI AND D. SULITZEANU

From the Department of Bacteriology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

In immunization experiments with living vaccines it is desirable to determine whether the microorganisms detected in the organs of the immunized and reinfected animals are descendants of the vaccinating or the superinfecting strain. The resistance against streptomycin was employed for the first time by Vaccarezza and Cetrangolo in 1950 in order to recognize the bacteria derived from primary and secondary tuberculous infections of guinea pigs.[1] In 1952 Olitzki utilized streptomycin-sensitive strains of *Brucella abortus* for primary and streptomycin-resistant strains for superinfections in white mice and vice versa.[2] Furthermore, a streptomycin-dependent mutant was employed as immunizing agent.[2,3] Pollack et al used a similar method in immunization experiments on guinea pigs.[4] The primary infection was accomplished by *Br. abortus* resistant to streptomycin, and the superinfection by *Brucella suis* sensitive to streptomycin.

The present paper deals with a method for the quantitative determination of the number of brucellae resulting from the primary and secondary infection under different experimental conditions.

MATERIALS

Mediums.—Trypticase-soy medium, a product of the Baltimore Biological Laboratory, was employed. Three percent of glycerol and 0.1 mg per liter of thiamine were added.

Strains.—The streptomycin-sensitive and the corresponding streptomycin-resistant strains (*abortus 19* and *2308*, respectively) were the same as described previously.[5] In addition to these strains an *abortus 19* strain was isolated from a lyophilized vaccine manufactured by the Lederle Laboratories, Pearl River, New York (serial no. 6241-172A, date of expiration Nov. 1, 1951).

Experimental animals.—Since preliminary experiments showed no sexual differences in resistance to infection, equal numbers of male and female mice were employed in all experimental groups. Marked differences, however, were observed in the course of the infection between animals of different ages, and the weights of the spleen and the liver, the main organs under observation, varied with increase of body weight, as shown in table 1. Six-week-old mice, weighing about 20 g, were therefore utilized in each experimental group. The absolute weights of the spleen and the liver varied markedly during the period of growth, but their relative weights remained below 0.8 per 100 g of body weight. A relative spleen weight exceeding the upper limit of 0.8% was therefore considered as a pathological feature.

Received for publication July 8, 1953.

1. Vaccarezza, R. F. and Cetrangolo, A. 1950, La sobreinfeccion tuberculosa experimental en animales tratados con estreptomicina. Arch. Tissol. **5**: 551–560.
2. Olitzki, A. L. 1952, The use of streptomycin-resistant and dependent mutants of *Brucellae* in immunization experiments. Symposium on basic medical Research, Jerusalem, June 9–11, 1952. Bull. Res. Counc. Israel, **2**: 325.
3. Olitzki, A. L. and Szenberg, E. 1953, Toxicity, infectivity and antigenicity of a streptomycin dependent mutant of *B. abortus* (strain 19). Proc. Soc. Exper Biol. & Med. **82**: 539–541.
4. Pollack, A. D., Kelly, E. H., Gorelick, A. N., Braun, W. and Victor, J. 1952, Superinfection in experimental brucellosis of guinea pigs. J. Infect. Dis. **30**: 267–272.
5. Olitzki, A. L. 1952, The determination of the most probable numbers of streptomycin-fast cells in Brucella cultures and their variability in growing and aging cultures. J. Gen. Microbiol. **6**: 166–174.

TABLE 1.—*Absolute and relative weights of liver and spleen in normal white mice of different ages (10 to 15 mice in each group).*

Age in weeks	Weight in grams	Weight of the		Relative weight per 100 g. of body weight:	
		Liver (g)	Spleen (mg)	Liver (%)	Spleen (%)
3	11.1	0.55	56	4.9	0.50
4	16.3	1.00	96	6.1	0.59
5	20.6	1.23	107	6.0	0.52
6	22.5	1.51	120	6.7	0.53
8	25.0	1.56	145	6.2	0.58
10	27.3	1.85	209	6.8	0.77
11	29.8	1.79	190	6.0	0.64
12	30.3	2.29	194	7.6	0.64

METHODS

Preparation of bacterial suspensions for infections and superinfections.—The growth of 48-hour cultures on agar was washed off with broth, and a bacterial suspension was prepared which effected a 50% light transmission at a wave length of 650A and contained 3.2×10^9 brucellae per ml.

From this suspension further dilutions in broth were prepared until the infecting dose was contained in 0.5 ml. The bacteria were administered intra-abdominally. Suitable dilutions were poured into plates and the infecting dose was checked by plate counts.

Determination of the number of brucellae in the infected organs.—After suitable time intervals, groups of five mice were killed by ether and weighed together. The organs were removed under sterile conditions, placed on Petri dishes and weighed in groups of five. Then five whole organs were ground with glass powder in porcelain mortars and the homogenized suspension of fine particles was taken up in 50 ml of broth. It was important that whole organs be triturated and cultured. Some residual infections were not detected when 0.2 ml of the organ suspension was spread out on plates but only when the triturated total organs were suspended in broth.

From this suspension further tenfold dilutions were made. From suitable dilutions, 0.2 ml were plated and the average number of brucellae per organ among a group of five calculated by plate counting, after an incubation of 10 days at 37 C. The ratio between the numbers of colonies grown on plates containing 100μg of streptomycin per ml and the number of colonies on the plates without streptomycin indicated the percentage of sensitive and resistant cells in the culture.

RESULTS

Since the differentiation between primary and secondary infections in the immunization experiments was based entirely on streptomycin resistance, experiments were performed in order to determine the back mutation rate in vivo from streptomycin-resistant to nonresistant organisms and vice versa. Fifteen mice were infected with *Br. abortus 19* resistant to streptomycin, and dissected after 30 days. From the spleens of all animals only streptomycin-resistant organisms were isolated. Similar results were obtained with streptomycin-resistant *Br. abortus 2308*, *Brucella melitensis* and *Br. suis*. A total of 65 mice were examined 30 to 41 days after the start of the infection. All strains remained streptomycin-resistant after this period and no instance of back mutation to nonresistance was observed.

Mutation of nonresistant to resistant bacteria was examined by a method described elsewhere.[5] One hundred ten cultures from spleens infected with strain *19* were examined—20 after 14 days, 30 after 30 days, 30 after 45 days and 30 after 60 days from the beginning of the infection. No significant differences in the appearance of streptomycin-resistant mutants were observed. The following mutation rates were observed at a level of 100 μg per ml:

One resistant bacterium per population of 1.0×10^8 to 4.9×10^8: 15 cultures

One resistant bacterium per population of 5.0×10^8 to 9.9×10^8: 20 cultures

One resistant bacterium per population of 1.0×10^9 to 4.9×10^9: 30 cultures

One resistant bacterium per population of 5.0×10^9 to 9.9×10^9: 45 cultures

In another experiment 80 mice were infected with nonresistant strains of *Br.*

abortus and dissected at different times between the 30th and the 40th days after the onset of the infection. When the spleens were cultured on plates containing 100 μg per ml, no growth of resistant colonies was observed. This result conformed with result of the former experiments. Since the mutation rate for the resistant mutants is at least 1 per population of 10^8 and the number of microorganisms in the spleen never exceeds 10^7, the possibility that resistant mutations will appear in the spleen and overgrow the nonresistant bacteria is very slight.

Since the mouse is considered by some workers as unsatisfactory for brucella research due to its high innate immunity to brucellae, experiments were carried out for the purpose of determining the normal course of the infection in nonimmune mice. With varying quantities of resistant and nonresistant strains *2308* and *19*, experiments showed that relatively small intra-abdominal inoculums were able to produce prolonged infections lasting for more than 1 month in the majority of the animals. Table 2 illustrates this.

The figures in table 2 show that an infective dose of 5×10^7 bacteria of strain *19* as well an infective dose of 5×10^6 bacteria of strain *2308* represented 10,000 MID or about 100,000 ID_{50}, when the state of infection after 14 days was taken into account. The experiments above revealed only the number of infected and noninfected mice but did not assess the severity of the infection. To determine severity the number of bacteria isolated from liver and spleen were counted by the method described above.

TABLE 2.—*The persistence of microorganisms in white mice after the intra-abdominal infection with cultures of* Br. abortus *strains* 19 *and* 2308.

Infecting dose	Percentage of infected animals after:			
	14 days	30 days	45 days	60 days
Strain *19*				
5×10^1 bacteria	0	20	0	0
5×10^3 bacteria	56	60	10	20
5×10^5 bacteria	100	90	20	10
Strain *2308*				
5×10^1 bacteria	65	60	82	20
5×10^3 bacteria	100	90	80	70
5×10^5 bacteria	100	90	80	70

When the counting method was employed for individual mice, it was found that the number of microorganisms isolated varied from animal to animal. A group of 9 mice was infected with 3×10^4 bacteria of strain *2308*. Twelve days later individual spleen counts showed the following results:

5.0 to 9.9×10^4 in 1 mouse
1.0 to 4.9×10^5 in 5 mice
5.0 to 9.9×10^5 in 1 mouse
1.0 to 4.9×10^6 in 2 mice

In another group infected with the same dose of the same strain, the spleens were cultured individually and after 27 days the results were:

1.0 to 9.9×10^4 in 1 mouse
1.0 to 9.9×10^5 in 3 mice
1.0 to 4.9×10^6 in 6 mice

The difference became smaller when the spleens of 5 mice were pooled and cultured together. A group of 30 mice was divided into 6 subgroups. The animals were infected with strain *2308* and sacrificed 7 days after the infection. The counts of the pooled spleens in the 6 groups were 5.0, 1.2, 3.7, 2.9, 2.4 and 6.4×10^5. The average was 3.6×10^5.

Another group of 20 mice was divided into 4 subgroups and dissected on the 30th day after the onset of the infection. The counts obtained in the 4 groups were 2.5, 1.9, 1.0 and 4.2×10^6. The average was 2.4×10^6.

These experiments demonstrated that pooled cultures from groups of at least 5 mice decreased the deviations which occurred with cultures from single organs.

The course of the infection in non-

TABLE 3.—*Average concentration of brucellae in the spleen and liver after the intra-abdominal infection with different stock cultures of* Br. abortus *strains* 2308 *and* 19.

Infecting *Br. abortus* strain	Infecting dose $\times 10^8$	Organ examined	Average number (10^8) of brucellae counted after different time intervals (days)							
			7	15	30	45	60	90	120	160
2308A	5.0	Spleen	16.5	1.0	47.0	6.4	2.8		0.04	0.05
2308B	5.0		0.2	4.0	4.2		1.0	0.01		
19A	500.0		5.4	22.0	0.04	0.005	0.0006	0		
	5.0		10.0	0.4	0.8	0.008	0.0002	0		
	0.05		20.0	43.0	0.001	0.0002	0			
19B	25.0		41.0	17.0	0.2	0.004	0.0005	0		
19C	3.0		5.6	5.7	0.001	0.001	0.0002	0		
2308A	5.0	Liver	0.5	0.3	0.8	0.4	0.4		0.002	0.08
2308B	5.0		0.03	0.2	0.01		0.005	0.002		
19A	500.0		0.1	0.06	0.003	0.001	0.002	0		
	5.0		0.1	0.008	0.2	0.001	0.005	0		
	0.05		0.01	0.9	0.0001	0.0001	0			
19B	25.0		0.1	0.3	0.03	0.0007	0.0006	0		
19C	3.0		0.0009	0	0					

immunized mice was followed by using the method of pooled organs. Table 3 summarizes some of the experiments performed with several nonresistant strains *19* and *2308*. These were derived from the same parent strain and simultaneously subcultured in the laboratory.

The results showed that strain *2308* persisted for a relatively long time in the spleen. Thirty days after infection more than 10^6 microorganisms were counted in the spleen. The 100- to 1000-fold decrease in bacterial count during this period indicated increasing immunity, and it was observed to occur repeatedly 7, 15 and 30 days after inoculation.

With strain *19*, however, 10^6 bacteria were counted in the spleen only during the first 14 days of infection, no matter what the size of the inoculum. At 30 days, even with an inoculum of 5×10^8, the bacterial count was always less than 10^6. When strain *19* was used as a challenge strain, only the observations obtained during the first 14 days after infection were considered significant.

No differences in behavior were noted between the streptomycin-resistant strain *19* and its parent nonresistant strain. Infection with resistant strain *2308* during the first month yielded lower bacterial organ counts than its parent nonresistant strain. The average organ count of 2×10^5 was sufficiently high for comparisons with the changes in counts of the reinfecting strain.

The relative spleen weight was of little significance. During the first month of the infection the spleen was enlarged beyond 1% of the body weight. During the second month the spleen gradually approached normal weight, although the infection continued. It was concluded that an enlarged spleen indicated infection, but a spleen of almost normal weight did not exclude the presence of infection.

The streptomycin-resistant and nonresistant brucella strains produced a similar infectious course in mice. Consequently, in the following experiments resistant and nonresistant strains were employed in order to study the effect of a primary infection on a superposed secondary infection. When the primary infecting strain was streptomycin-resistant and the secondary strain nonresistant or vice versa, it was possible to observe precisely the course of the primary and the superposed infection by the quantitative determination of both types of brucellae.

Figures 1 and 2 show the effect of primary infections on reinfections performed 26 days later.

When the primary infection was produced by the nonresistant strain *19* and the secondary infection by the strepto-

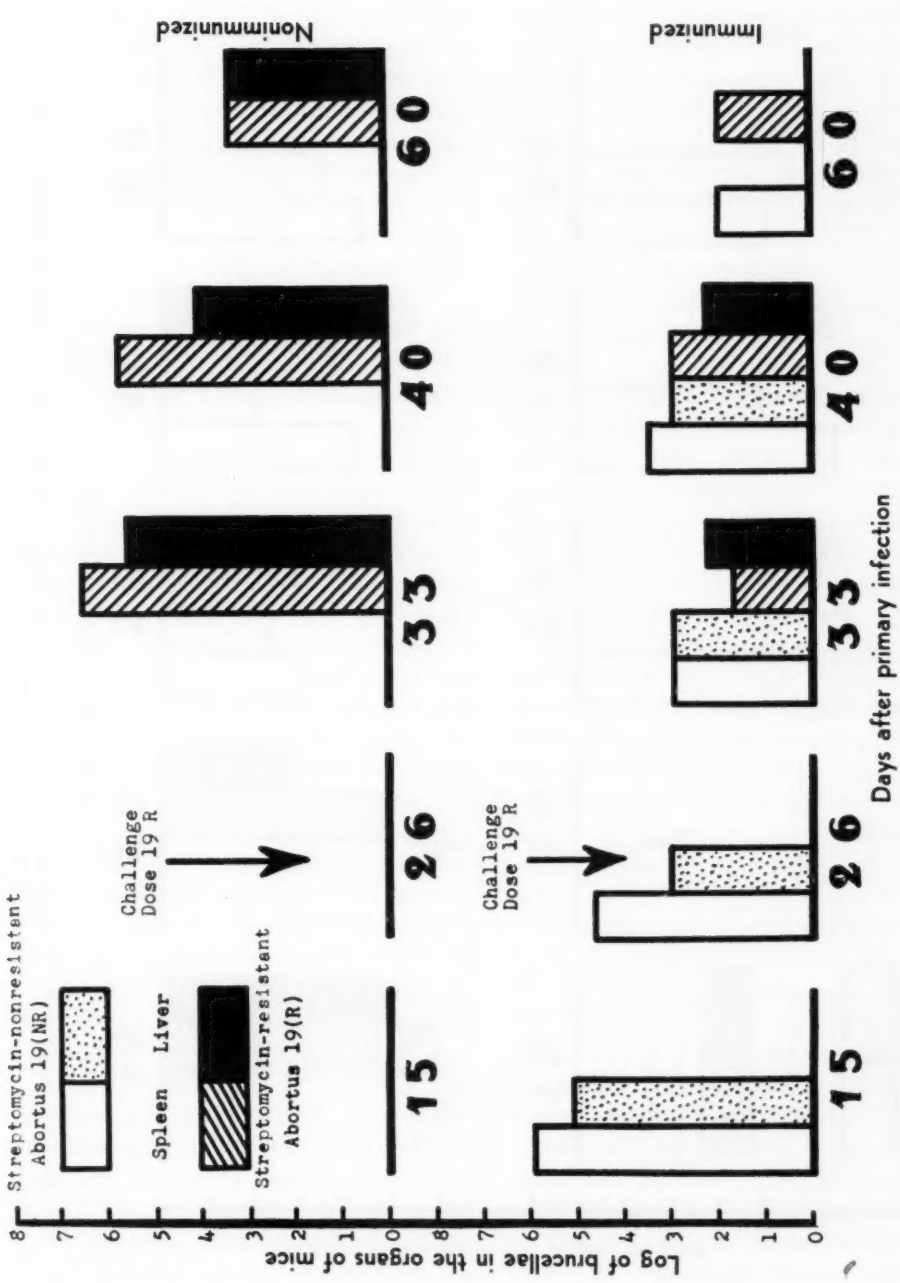


FIG. 1.—Distribution of brucellae in the organs of infected mice. Immunization by living *Br. abortus 19* (NR); 26 days later superposed infection with *Br. abortus 19* (R).

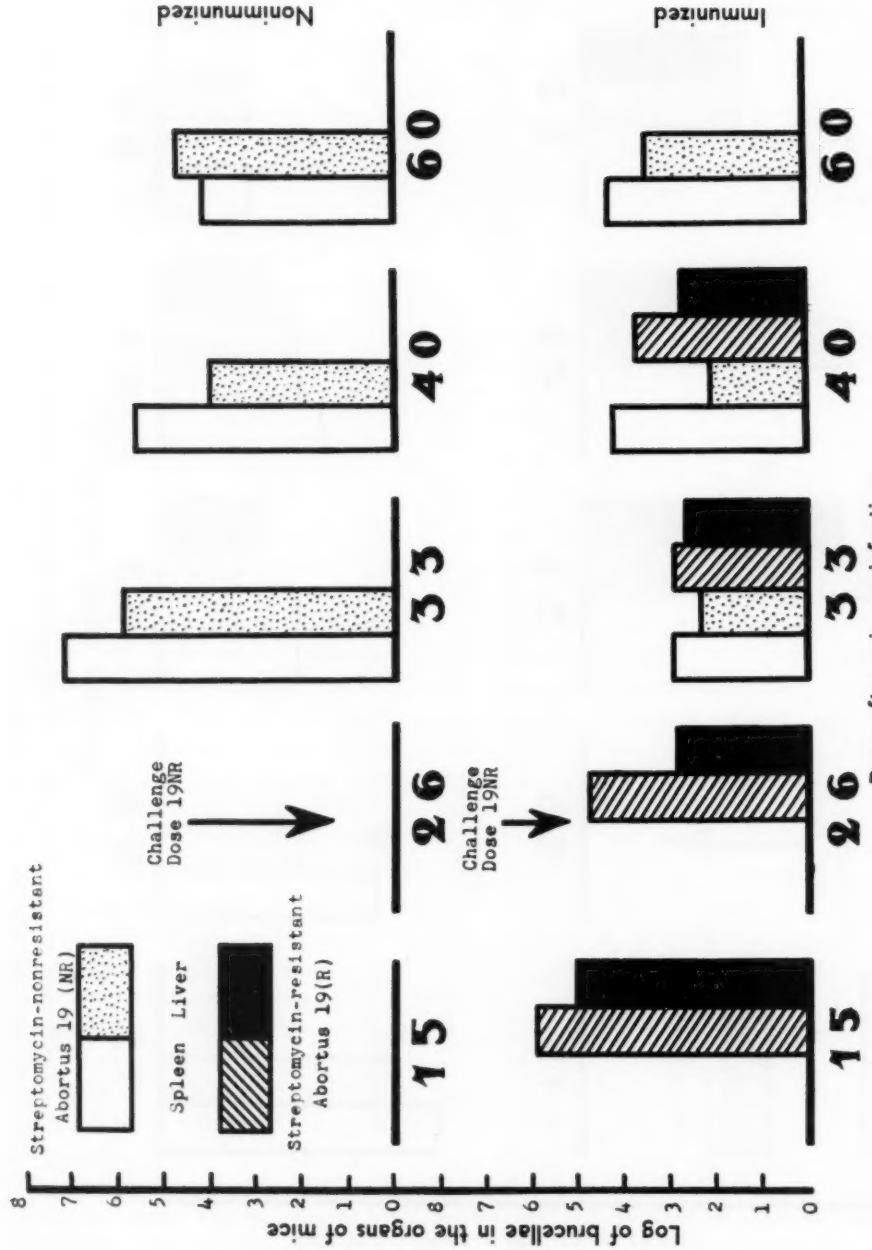


FIG. 2.—Distribution of brucellae in the organs of infected mice. Immunization by living *Br. abortus 19* (R); 26 days later superposed infection with *Br. abortus 19* (NR).

mycin-resistant strain *19* (fig. 1), the secondary infection was unable to progress in the already infected organ. The primary infection, already in regression, continued to decline, and on the 60th day after its onset the liver was completely sterile and the spleen contained some hundred organisms. The relative spleen weight decreased from the 15th day after the start of the primary injection and continued to decrease even after the second injection.

When the sequence was reversed (fig. 2) and the primary infection was initiated by the streptomycin-resistant strain *19*, then a limited secondary infection by the nonresistant strain *19* was observed. Thirty-four days later, 17,000 nonresistant organisms were found in the spleen and 2300 in the liver, while the primary streptomycin-resistant strain had disappeared. The secondary infection, however, did not produce a secondary enlargement of the spleen and the maximal concentration of organisms found in the spleen and liver was considerably less than that observed in the nonimmune animals, particularly in the early period of the reinfection.

Figures 3 and 4 show the results of reinfections superposed on the 47th day after the onset of the primary infection, when the organisms remaining from the primary infection had disappeared. In both these experiments only organisms of the superposed infections were found.

After primary infection with the nonresistant strain *19* (fig. 3), the superimposed infection by the streptomycin-resistant strain *19* was limited. The spleen weight was not enhanced, and brucellae were not present in the liver. Their maximal number in the spleen was about 400 times less than that observed in the untreated controls and dropped to nearly zero within a month.

When the secondary infection with the nonresistant strain was superposed (fig. 4) on the 47th day after the onset of the primary infection, then, 12 days later, the spleen weight was enhanced and the concentrations of brucellae in spleen and liver were nearly the same as in the untreated control animals. However, a difference between the immunized animals and the untreated controls was observed on the 34th day after the onset of the superposed infection. At this time the organs of the immunized mice were almost free of brucellae, while organs of the controls harbored about 1000 organisms on the average.

In all these experiments the livers of both untreated and immunized animals contained fewer organisms than the spleens. In doubly infected animals the liver acquired a complete immunity, i.e., inability to harbor the microorganisms, while the spleen still contained them.

In order to exclude the possibility of errors which might have arisen as a result of the relatively rapid disappearance after infection of streptomycin-resistant and nonresistant strains *19*, the more persistent strain *2308*, resistant to streptomycin, was used as challenge strain in the following experiments.

Two vaccines were employed as primary inoculations: (a) living *Br. abortus 19*, recently prepared from a stock culture nonresistant to streptomycin (Jerusalem); (b) a lyophilized strain *19* vaccine prepared before 1951 in the Lederle Laboratories, Pearl River, New York and kept until the start of the experiment, October 1952, in the refrigerator. It contained 1% living brucellae.

The potency of these vaccines was tested by the mucin method similar to that described by Olitzki and Kushnir.[6]

6. Olitzki, A. L. and Kushnir, R. 1952, The immunizing potency of living *Brucella abortus* vaccines tested in lethal infections of white mice produced with the aid of the mucin technique. Bull. Res. Coun. Israel, 2: 199–201.

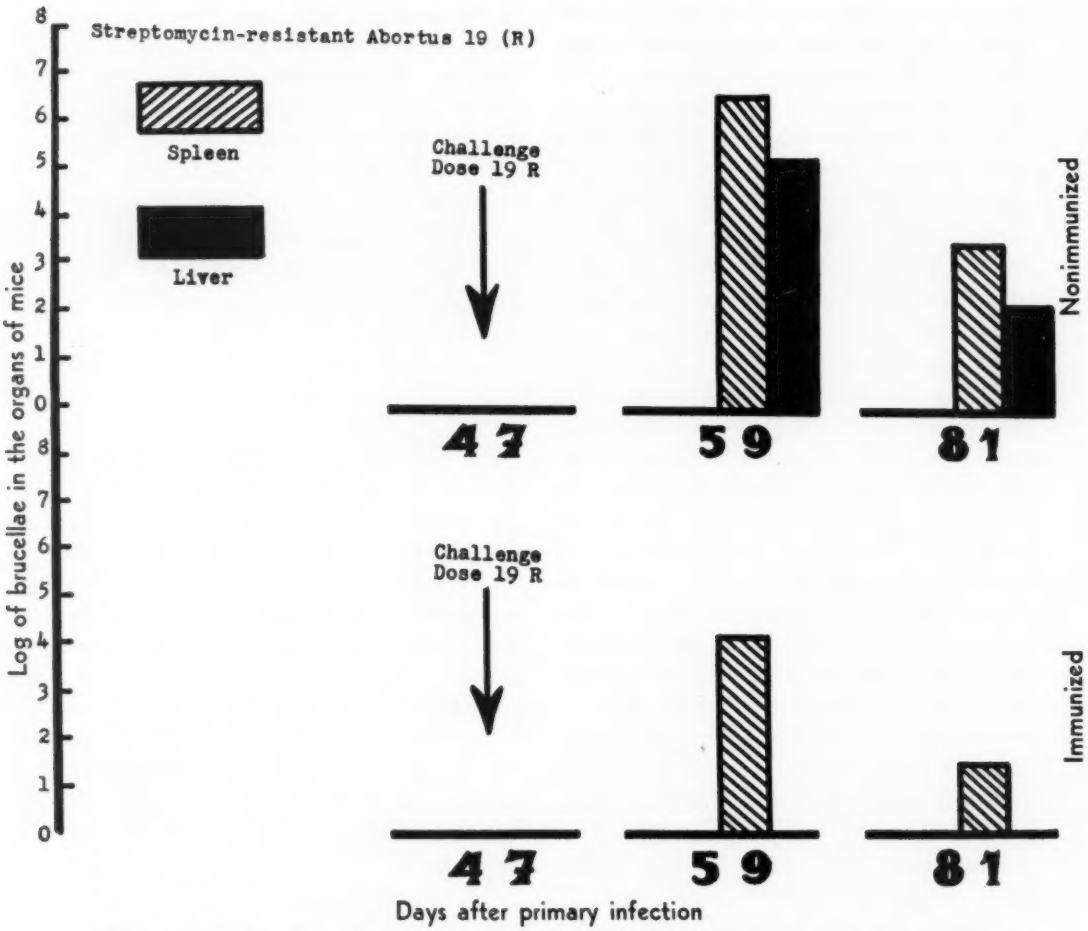


FIG. 3.—Distribution of brucellae in the organs of infected mice. Immunization by living *Br. abortus 19* (NR); 47 days later challenge injection by *Br. abortus 19* (R); the primary infection has disappeared.

A challenge dose of 10^9 bacteria of strain *2308* suspended in 0.5 ml of a 5% mucin solution was given 30 days after the immunizing injection. In nonimmunized mice this dose of strain *2308* produced a 100% mortality.

The mortality figures as summarized in table 4 show that the lyophilized vaccine was nearly as effective as an acetone-killed vaccine, and the immunizing potency of the recently prepared living vaccine surpassed both of them over 100-fold. If, however, the immunized animals received a sublethal challenge dose, i.e., 10^5 bacteria of strain *2308* without mucin, and quantitative examinations of the bacterial content of the spleen were carried out, results were obtained as summarized in table 5.

Table 5 shows that doses of 10^8 and 10^6 lyophilized vaccine, containing only 1% living brucellae, were able to prevent almost completely the invasion of the spleen by the secondary streptomycin-resistant bacteria. The newly prepared vaccine, containing almost 100% living bacteria, was unable to exert such an effect. Seven days after the reinfection, the bacterial spleen count of the reinfecting streptomycin-resistant strain

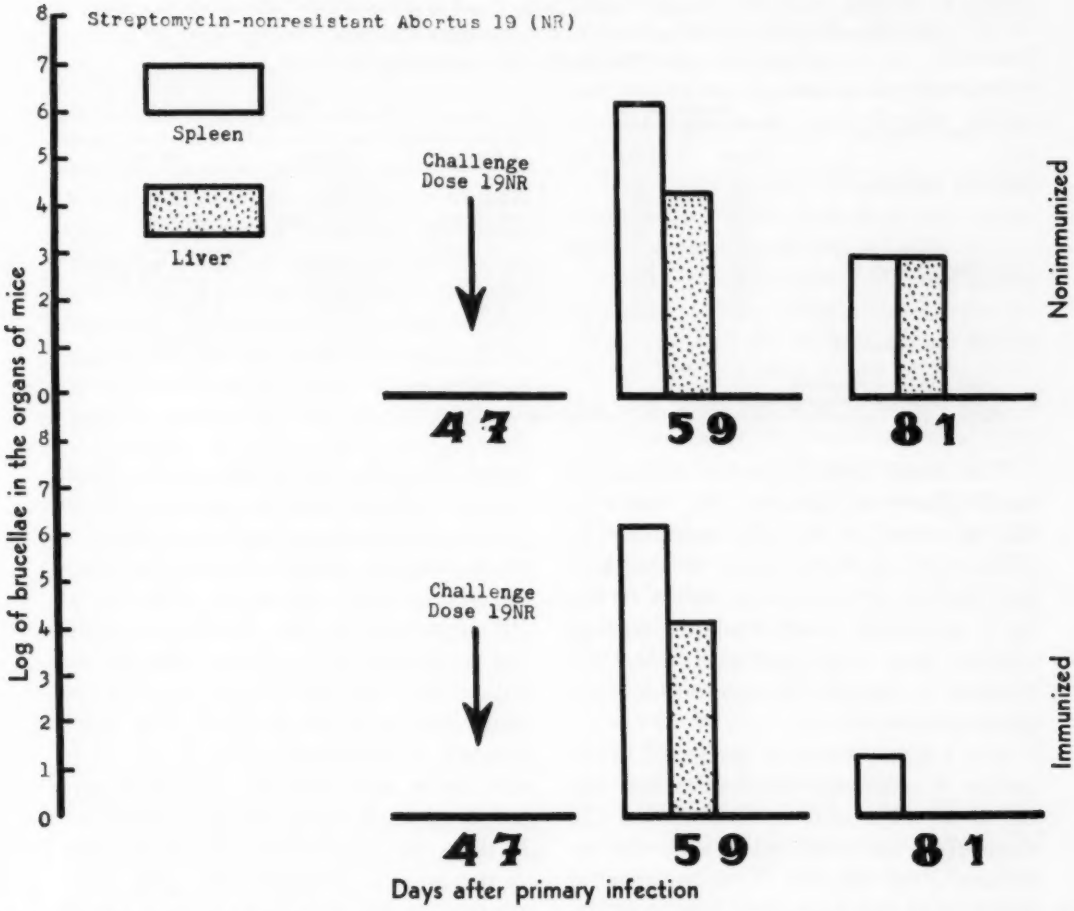


FIG. 4.—Distribution of brucellae in the organs of infected mice. Immunization by living *Br. abortus 19* (R); 47 days later challenge injection by *Br. abortus 19* (NR); the primary infection has disappeared.

2308 was 500 to 5000 times less than in the nonimmunized controls and these microorganisms persisted. Thirty days after the reinfection they were still found in the spleen.

TABLE 4.—*Mortality in groups of 10 mice immunized with varying quantities of different types of vaccines after a challenge dose of 10^9 brucellae* (A2308) *suspended in mucin.*

Type of vaccine	Deaths observed in the groups immunized by 1 injection of the following quantities of brucellae:				ED_{50}
	10^2	10^4	10^6	10^8	
Living, recently prepared	7	5	4	1	$10^{4.3}$
Lyophilized, 1% living	9	8	8	3	$10^{5.8}$
Acetone-killed	9	7	10	0	$10^{5.4}$

Number of deaths in the nonimmunized controls: 10. Observation time: 14 days.

The following experiment was carried out in order to determine whether the immunizing effect of the lyophilized Lederle vaccine was due to its content of living and dead bacteria, or whether the strain *19* employed for its preparation had properties different from those of the Jerusalem strain. The surviving bacteria of the lyophilized vaccine were isolated and the immunizing potency of these living organisms was compared with that of the living Jerusalem strain *19*.

TABLE 5.—*Number of brucellae (resistant strain 2308) found at different time intervals in the spleens of mice immunized by varying quantities of different types of vaccine. Challenge dose: 10^6 brucellae given 30 days after the immunizing injection.*

Type of vaccine	Number of immunizing bacteria		Number and type of brucellae in the spleens after the reinfection		
	Total	Living	7 days	14 days	30 days
Strain 19, NR, living	10^8	10^8	4×10^3 R	2×10^3 R	7×10^3 R
	10^6	10^6	2×10^3 R	6×10^3 R	5×10^3 R
	10^4	10^4	2×10^3 R 60%	2×10^4 R 80%	6×10^3 R
	10^2	10^2	2×10^3 R	3×10^3 R	7×10^4 R
Lyophilized vaccine, 1% living, NR	10^8	10^8	1×10^5 NR	5×10^4 NR	3×10^4 NR 90%
	10^6	10^4	2×10^5 NR	5×10^3 NR	1×10^3 NR 50%
	10^4	10^2	5×10^4 R	3×10^3 R	1×10^4 R
	10^2	10^0	2×10^4 R	2×10^4 R	1×10^4 R
Controls, not immunized			1×10^6 R	2×10^4 R	1×10^4 R

NR = 100% nonresistant.
R = 100% streptomycin-resistant.
R 60% = 60% streptomycin-resistant and 40% nonresistant.

The mucin test did not reveal significant differences between the immunizing potencies of the two vaccines. The ED_{50} of the Lederle strain *19* was $10^{4.0}$ and that of the Jerusalem strain *19* was $10^{4.4}$. However, when the quantitative method was employed, significant differences in the immunizing potency appeared (table 6).

The Lederle strain *19* persisted in the spleen in relatively large numbers, 10^4, up to 60 days after its inoculation (30 days after the reinfection with the resistant *2308* strain). The number of bacteria of the Jerusalem strain *19* decreased to 10^2. The Lederle strain effected a more marked splenic enlargement than the Jerusalem strain. Both strains, administered in doses of 10^6 to 10^{10}, were able to prevent completely a reinfection by the streptomycin-resistant strain *2308*. An initial infection of 10^4 organisms of the Jerusalem strain did not persist 30 days after inoculation with strain *2308* and did not prevent the reinfection with strain *2308*. The same number of microorganisms of the Lederle strain as a primary infection persisted up to 60 days and prevented for 30 days the appearance of strain *2308* in the spleen. However, 30 days after reinfection, 10% of the organisms found in the spleen were of the resistant strain.

TABLE 6.—*Number of brucellae in the spleens of mice immunized with two different streptomycin-sensitive strains* (abortus 19) *and reinfected 30 days later with 10^4 bacteria of the streptomycin-resistant strain* 2308.

Type of living vaccine	Number of bacteria	Number and type of brucellae in the spleens after the reinfection			Percentage of spleen weight after the reinfection		
		7 days	14 days	30 days	7 days	14 days	30 days
Abortus 19, Jerusalem, NR	10^{10}	5×10^3 NR	3×10^3 NR		1.2	0.9	
	10^9	2×10^4 NR	3×10^3 NR	1×10^3 NR	1.5	0.9	1.4
	10^8	1×10^3 NR	2×10^3 NR	3×10^3 NR	1.0	1.0	0.9
	10^6	1×10^3 NR 80%	2×10^3 NR	1×10^3 NR	1.0	0.9	0.8
	10^4	2×10^3 R	1×10^3 NR	1×10^3 R	0.8	0.8	0.9
Abortus 19, NR, from lyophilized vaccine	10^{10}	3×10^5 NR	3×10^5 NR		2.2	1.5	
	10^8	1×10^5 NR	2×10^5 NR	9×10^4 NR	1.6	1.3	1.3
	10^6	3×10^4 NR	4×10^4 NR	4×10^4 NR	1.9	1.2	1.0
	10^4	2×10^5 NR	5×10^4 NR	1×10^4 NR 90%	1.0	2.2	0.7
Control, not immunized		5×10^4 R	5×10^4 R	4×10^4 R	1.0	0.7	1.3

NR = 100% nonresistant.
R = 100% streptomycin-resistant.
NR 90% = 90% nonresistant and 10% resistant.

DISCUSSION

The quantitative method described above permitted the elucidation of several problems arising from the use of mice for immunization in experimental brucellosis. It is generally accepted that white mice are less susceptible to brucella infection than guinea pigs, and that the infection of the mouse is retrogressive.[7] A retrogressive type of infection was found in mice inoculated with strain *19*, but the infection with strain *2308* persisted at least 6 months. It seemed, therefore, that strain *2308* as a reinfection constituted a better challenge for quantitative immunization experiments than strain *19*.

Living vaccines prepared from *abortus 19* strains of different origin showed varying degrees of immunizing potencies, ranging from the ability to reduce the number of the reinfecting organisms to complete prevention of their appearance in the spleen. The outcome of the immunization experiments depended on several factors:

1. The origin and the dosage of the immunizing strain.—The experiment presented in table 6 shows that two living *abortus 19* strains of different origin in dosages of 10^6 or more were able to prevent completely a reinfection. They differed in their immunizing effect when 10^4 microorganisms were administered. The Jerusalem strain did not persist and was immediately replaced by the reinfecting streptomycin-resistant strain. Although a long-persisting strain gave a better immunity in this experiment, it could not be concluded that the survival of the immunizing strain was necessary to effect immunity. The experiment presented in figures 3 and 4 shows that even when the primary infecting (immunizing) strain has disappeared at the time of the reinfection, the number of reinfecting microorganisms remained limited, in comparison with the nonimmunized controls, and finally disappeared.

This latter result was similar to that obtained by Pollack et al in immunization experiments on guinea pigs.[4]

2. The infected organ.—In most of the experiments the number of microorganisms found in the spleen surpassed by 10- or 100-fold that found in the liver. All the other organs contained only a few hundred or thousand brucellae from the beginning of the infection and were therefore not included in this study. It became clear that under these circumstances it was easier to obtain a complete immunity, i.e., the inability to store the reinfecting organisms, in the liver than in the spleen. In the experiments presented in figures 1 to 4 the liver was sterile on several examinations, while the spleen continued to harbor the reinfecting microorganisms.

3. The reinfecting strain.—The outcome of the experiments also depended on the nature of the reinfecting microorganisms. When microorganisms that cause a retrogressive infection, such as Jerusalem strain *19*, were used as reinfecting bacteria, the number of reinfecting organisms decreased in both the immunized and control animals and approached zero in the immunized mice (fig. 3 and 4). On the other hand, the reinfecting strain, such as *2308*, showed a tendency to persist for a long time in the spleen. As the primary infection regressed, some of the reinfecting organisms survived and finally replaced the vaccinating bacteria. Examples of this replacement are presented in tables 5 and 6.

The dosage of the reinfecting strain was also important. A reinfecting dose of 10^5 organisms resulted in invasion of

7. Wilson, G. S. and Miles, A. A. 1946, Topley and Wilson's Principles of Bacteriology and Immunity ed. 3, vol. 1, p. 829.

the spleen following immunization by 10^4 to 10^6 living bacteria of the Lederle strain (table 5). A reinfecting dose of 10^4 organisms was completely prevented from invading the spleen following immunization by the same quantity of Lederle microorganisms (table 6).

4. The culture method.—Both culturing of whole organs on a fluid medium and the plating method were necessary to detect small residual infections. The determination of the percentage of infected animals as accomplished by Pollack et al[4] did not reveal the extent of infection within the organs. Their method was not capable of showing whether ten or millions of microorganisms were present in the infected organs. Furthermore, the use of only portions of organs was not sensitive enough to isolate the invading bacteria. Whole organs must be cultured for accurate quantitative results.

SUMMARY

In experimental brucellosis in white mice the optimal immunizing effect was obtained by using a quantity of living organisms sufficient to survive at the time of reinfection and capable of inhibiting completely the invasion of the reinfecting microorganisms into the spleen. When the primary infecting strain, however, disappeared from the infected organs at the time of reinfection there resulted a state of relative immunity in which the number of reinfecting microorganisms in the spleen remained limited and finally disappeared.

AGE AND SEX AS FACTORS INFLUENCING *PLASMODIUM BERGHEI* INFECTIONS IN INTACT AND SPLENECTOMIZED RATS

AVIVAH ZUCKERMAN AND MEIR YOELI

From the Department of Parasitology, The Hebrew University, Jerusalem, Israel

In a study comparing *Plasmodium berghei* infections in intact and splenectomized albino laboratory rats, significant fluctuations were observed in the severity and duration of infections in different groups of intact controls. Both the age and the sex of intact rats were scrutinized as factors possibly leading to the observed fluctuations. The present paper summarizes experiments of this type, and compares infection courses in intact and splenectomized rats selected according to age and sex.

MATERIALS AND METHODS

Bartonella-free white rats were employed, from a colony maintained at the Hebrew University for the past 25 years. Ages and weights varied, as designated in each experiment. The strain of *P. berghei* was kindly provided by Professor H. E. Shortt, F. R. S., of the London School of Hygiene and Tropical Medicine, and has been maintained for several years by blood passage in white mice. Inoculations were given intraperitoneally and consisted of a standard dose of 40 million infected rat or mouse erythrocytes, collected during the acute rise of the infection to avoid possible antibody effects occurring at or after crisis. In the experiment on suckling rats, however, 10 million rather than 40 million parasitized red cells were employed as inoculum. Giemsa-stained films of tail blood were examined daily, and the number of parasitized cells per 10,000 red cells was recorded.

Conditions under which the experimental animals were maintained did not vary appreciably during the observation period.

Received for publication August 19, 1953.

The authors are grateful to Professor Saul Adler and Professor Bernhard Zondek for their advice and criticism.

RESULTS

1. The influence of age on the course of P. berghei *infections in intact rats*

Five groups of rats of varying age infected with *P. berghei* serve to illustrate the development of a clear age immunity to this parasite in the absence of previous exposure. Data on their infection courses are compared in table 1.

Two groups of rats whose exact ages were not on record appear in the table in the order of increasing weights, which were considered to be a function of increasing age.

Immunity increased with age, as reflected in the progressively milder parasitemias and lower mortality rates recorded with advancing age, as well as in the fact that crisis occurred earlier in older rats. While a certain increase in immunity was already observed in rats weighing 66 g as compared with the

TABLE 1.—P. berghei *infections in albino rats of varying age.*

Age (days)	Number of rats	Weight in g when infected	Mean day of peak parasitemia	Mean percent peak parasitemia	Mortality rate (%)	Mean day of death	Days of microscopically demonstrable patency before latency
14 to 17*	32		15.2±0.19	98.5±0.41	30/32 (94)	17.0±0.23	21.5±0.11
30	24	29.2±0.24	15.0±0.60	93.7±1.27	24/24 (100)	15.9±0.60	
	11	66.5±5.40	17.3±1.51	76.1±7.79	7/11 (64)	21.6±1.24	28.5±3.13
	22	120.2±2.50	8.0±0.36	8.2±2.31	6/22 (27)	10.1±0.25	20.5±0.82
114	18	187.0±7.29	8.3±0.77	5.9±1.66	6/18 (33)	10.3±1.05	25.3±2.56

* Suckling rats were infected with 10 million parasitized erythrocytes. All other rats received the standard inoculum of 40 million.

TABLE 2.—P. berghei *infections in male and female rats of three age groups. Group A (14- to 17-day suckling rats) received 10 million parasitized erythrocytes intraperitoneally. Groups B (30-day adolescents) and C (114-day mature rats) received standard inoculums of 40 million parasitized erythrocytes.*

Group		Number of rats	Weight in g when infected	Mean day of peak parasitemia	Mean percent peak parasitemia	Mortality rate (%)	Mean day of death in rats succumbing	Mean days of patency
A	Male	18		15.1±0.19	99.2±0.40	17/18 (94)	16.7±0.25	22
	Female	14		15.3±0.04	98.0±0.24	13/14 (93)	17.5±0.40	21
	Standard error of difference between means			0.42	0.86		0.47	
	P			0.64	0.19		0.12	
B	Male	12	28.7±1.10	14.1±0.96	90.8±2.33	12/12 (100)	14.7±0.87	
	Female	12	29.8±1.01	16.0±0.59	96.5±0.79	12/12 (100)	17.2±0.65	
	Standard error of difference between means		1.50	1.13	2.46		1.09	
	P		0.48	0.13	0.05		0.05	
C	Male	10	206.5±8.90	8.5±1.33	6.7±2.90	3/10 (30)	11.3±1.91	28.0±4.03
	Female	8	162.4±4.26	8.0±0.50	5.0±0.25	3/8 (37)	9.3±0.48	21.6±1.00
	Standard error of difference between means		9.86	1.42	3.12		1.97	4.16
	P		<0.01	0.74	0.60		0.42	0.18

two younger groups, the difference in susceptibility was most striking between the 66- and the 120-g group, i.e., between groups designated below as adolescent and young adult rats. In one important point, however, increasing age favored increasing susceptibility. In fatal cases older rats died earlier than did younger succumbing rats.

2. *The influence of sex on the course of* P. berghei *infections in intact rats*

Table 2 compares infections in males and virgin females in three age groups: suckling, month-old and adult rats, respectively. While the three groups differed significantly from one another, as discussed in section 1, males and virgin females of identical ages within each group reacted alike to *P. berghei*. The only point of difference that emerged from this analysis is the fact that mature virgin females are somewhat smaller than mature males of identical age which have been reared under identical conditions.

In standardizing our procedure we were careful to select virgin female rats, since we had reason to suspect that the recent production of young reduced the female's susceptibility. In 4 mature female rats which had each produced a litter within a month or less before exposure to *P. berghei*, mean peak percent parasitemias were 5.9±0.60; the peaks occurred 3.0±0.01 days after inoculation; latency began 9.8±0.22 days after inoculation, and all of the 4 survived (compare with data on virgin females in table 2, group C). Although the exact age of these rats is not recorded, they probably fell within the category of the oldest group in table 2, group C. If, as is possible, they were younger than the rats in that section, the difference between them and virgin females of similar age would tend to be even more pronounced.

Infections in females shortly after parturition were thus milder than those in virgin females, although peak parasitemias were similar. Their increased resistance was expressed in earlier crisis, shorter duration of patency and

lower mortality rates than in virgin females. While the experimental sample is admittedly small, the reactions of all the rats in this group were strikingly similar. The low mortality rate was particularly noteworthy, and was further supported by data on similar females in table 3. In all, 19 females were studied shortly after parturition, and only 1 of these died of the infection, whereas 3 out of 8 adult virgin females succumbed (cf. table 2).

In view of the suspicion raised by the above experiment that susceptibility to *P. berghei* might be associated with endocrine function, the effects of ovariectomy and mammary extirpation in females and of castration in males were further studied. Table 3 compares infections induced in 5 intact and 10 ovariectomized females, all shortly after parturition. The infections were induced 17 days after ovariectomy was performed. Ovariectomy did not increase susceptibility to *P. berghei*, as it might be expected to have done if ovarian secretion was the factor depressing susceptibility. Similarly, mammary extirpation in 6 lactating females and surgical castration of 5 adult males had no demonstrable effect on their susceptibility to *P. berghei*, as compared with appropriate intact controls of the same age and sex.

3. The effect of splenectomy on P. berghei *infections in rats*

P. berghei infections induced in splenectomized rats were compared with those in intact controls in three experiments (A, B and C), of which essential data are summarized in table 4. Although the exact ages of groups A and B were not on record, it is clear from their initial weights that rats in group A were approximately 5 weeks old, those in group B were about 3 months old (sexually immature adolescents), and those in group C were mature adults although not entirely full grown, since our strain of rat may attain a maximum weight of 200 to 250 g. Sex was not on record in group A; all the rats in group B were females; and group C consisted of 9 males and 7 virgin females (splenectomized), and 10 males and 8 virgin females (intact). Since it was shown in section 2 that sex did not affect susceptibility if the females were virgin, and since all females in groups A and B were sexually immature while those in group C were virgin, it was legitimate to consider groups A, B and C as differing essentially in the single variable, age. In each experiment initial weights (i.e., ages) of splenectomized rats did not differ significantly from those of intact controls.

Splenectomy before inoculation had an ultimately adverse effect on resistance of rats to *P. berghei*, although in several respects it seemed to favor resistance for a time. Mortality rates in all age groups were higher in splenectomized rats than in intact controls, but the extent of this difference was greater in younger rats than in the mature group. Mortality rates for all the ani-

TABLE 3.—P. berghei *infections following a standard inoculum in intact as compared with ovariectomized adult female rats, all having recently produced litters.*

	Number of rats	Mean day of peak parasitemia	Mean percent peak parasitemia	Mortality rate (%)	Mean days of patency
Ovariectomized	10	6.9±1.18	8.5±3.14	1/10 (10)	17.3±1.27
Intact	5	6.8±0.72	6.6±1.26	0/5 (0)	17.0±1.33
Standard error of difference between means		1.38	3.39		1.83
P		>0.90	0.60		0.88

TABLE 4.—P. berghei *infections in intact and splenectomized rats of three age groups (A, B and C).*

Group		Number of rats	Weight in g when infected	Mean day of peak parasitemia	Mean percent peak parasitemia	Mortality rate (%)	Mean day of death in rats succumbing	Parasites seen in surviving rats on day (x) + positive − negative
A	Splenectomized	9	78.3±5.24	17.6±2.42	16.4±1.78	8/9 (89)	23.5±1.54	1/1 +(57)
	Intact	10	68.9±5.39	16.9±1.62	74.4±8.39	6/10 (60)	21.5±1.45	4/4 −(57)
	Standard error of difference between means		7.52	2.91	8.58		2.11	
	P		0.25	0.82	<0.01		0.38	
B	Splenectomized	7	117.9±0.92	15.9±2.90	20.0±2.08	7/7 (100)	23.3±1.26	
	Intact	6	121.8±1.04	6.2±1.28	15.4±6.25	1/6 (17)	17	5/5 −(45)
	Standard error of difference between means		1.39	3.17	6.57			
	P		0.05	0.03	0.51			
C	Splenectomized	16	188.4±8.54	13.2±2.39	7.7±0.56	8/16 (50)	24.7±0.89	8/8 +(58)
	Intact	18	186.9±7.30	8.3±0.24	5.9±0.45	6/18 (33)	10.3±0.56	12/12 −(58)
	Standard error of difference between means		11.24	2.51	0.72		1.49	
	P		0.90	0.07	0.03		<0.01	

mals in table 4 and for an additional 32 rats in the youngest group (16 intact and 16 splenectomized) are summarized in table 5. No splenectomized rat surviving subsequent inoculation was capable of suppressing the infection to latency at a time when parasites had no longer been microscopically demonstrable in appropriate intact controls for many weeks. Indeed, in several such splenectomized rats parasites have been seen more than a year after infection.

Splenectomized rats of all ages behaved like very young intact rats in two respects. (1) Whereas crisis occurred progressively earlier as age advanced in intact rats, it occurred fairly uniformly at the time of crisis in young intact rats in all splenectomized rats irrespective of age. (2) Death was delayed to a certain extent in succumbing splenectomized as compared with intact rats, and all splenectomized rats died at the time when young intact rats died, while aging intact rats died progressively earlier.

TABLE 5.—*Mortality rates (percent) in 98 intact and splenectomized rats of varying age. (Figures in parentheses represent the the number of animals dying in each group.)*

Age of rats	Intact	Splenectomized
Young	46 (12/26)	96 (24/25)
Adolescent	17 (1/6)	100 (7/7)
Mature	33 (6/18)	50 (8/16)

An unexpected and as yet unexplained age difference in splenectomized rats was noted with respect to peak parasitemias. A clearly significant difference between peaks, favoring the splenectomized and not the intact animals, was noted in the youngest but not in other groups. As noted above, splenectomy favored the host in another way as well, i.e., by delaying death. Although young splenectomized rats more efficiently controlled parasitemia than intact controls, the intact state favored ultimate recovery in this as in other age groups. This experiment was repeated with 16 intact and 16 splenectomized rats of the youngest age group (weights 63.5±1.76 and 64.2±1.47 g, respectively), in order to check the validity of the observation that parasitemias were more severe in intact than in splenectomized young rats. A difference in the same sense was again obtained, although it was less marked than in the

first experiment done with the same strain 35 months previously (mean peak parasitemias: splenectomized, 22.6% ±2.40; intact, 35.5% ±4.21; standard error of difference between means, 4.85; $P=0.02$). Other results of this experiment essentially resembled those in table 4, group A. Since all 16 splenectomized rats succumbed while 10 of the 16 intact controls survived, partial suppression of parasitemia was again independent of protection.

In table 6 the infection courses of 98 rats are analyzed to determine whether (1) variations in age, (2) the presence or absence of a spleen or (3) a fatal outcome or recovery were correlated with the severity of an infection. This table is based on infections in categories A, B and C in table 4, together with the additional 32 infections used to confirm the results in category A, table 4.

Infections in table 6 were arranged roughly in the order of descending severity. *Fulminating* infections were those in which parasitemia advanced progressively towards a fatal terminal peak well above 20% and often above 90%, with possible minor fluctuations of short duration. Periods during which parasitemias remained relatively stationary (ca. 10% ±10%) for at least a week were designated "*plateaus*." If a plateau period occurred initially, followed by a fatal terminal rise, the infection was considered to be slightly less severe than a fatal fulminating infection rising steeply from the beginning. Next in the order of descending severity were infections beginning with a plateau, interrupted by a peak and crisis followed by a chronic plateau terminating either in death or recovery. The least severe infections were low-grade plateaus throughout, with no well-defined crises, and terminating either in death or recovery.

The ability of the intact rat to cope adequately with infection increased with age. The only fatal, fulminating infections observed occurred in younger groups. Although some young, intact rats were already capable of controlling severe parasitemias after crisis or of maintaining subcritical levels throughout, the older the rat the more likely it was to be able so to control infection. Subcritical, plateau parasitemias did not, however, preclude death in mature intact rats.

Types of infection course varied less markedly with age if rats were splenectomized rather than intact when inoculated. All three age groups of splenectomized rats contained representative fatal infections, ranging from acute parasitemias in which crisis was never achieved to parasitemias subcritical

TABLE 6.—*Types of parasitemia course in 98 intact and splenectomized rats infected with* P. berghei. (*Figures in parentheses represent percentages.*)

Condition when infected		Type of parasitemia	Fulminating	Plateau to terminal peak	Plateau, peak, crisis, plateau	Plateau
Intact	Rats succumbing to infection	Young (A)	8/12 (67)	4/12 (33)		
		Adolescent (B)	1/1 (100)			
		Mature (C)				6/6 (100)
	Rats surviving infection	Young (A)			10/14 (71)	4/14 (29)
		Adolescent (B)				5/5 (100)
		Mature (C)			1/12 (8)	11/12 (92)
Splenectomized	Rats succumbing to infection	Young (A)		5/24 (21)	8/24 (33)	11/24 (46)
		Adolescent (B)		3/7 (43)	1/7 (14)	3/7 (43)
		Mature (C)		2/8 (25)		6/8 (75)
	Rats surviving infection	Young (A)				1/1 (100)
		Adolescent (B)				
		Mature (C)				8/8 (100)

TABLE 7.—P. berghei *infections in splenectomized male and female rats, 114 days old when inoculated.*

	Number of rats	Weight in g when infected	Mean day of peak parasitemia	Mean percent peak parasitemia	Mortality rate (%)*	Mean day of death in rats succumbing
Male	9	209.7±9.56	14.6±3.19	8.0±2.66	5/9 (56)	24.6±2.65
Female	7	161.1±6.34	11.4±3.44	7.4±2.62	3/7 (43)	25.0±4.97
Standard error of difference between means		11.47	4.69	3.73		5.63
P		<0.01	0.52	0.88		>0.90

* Infections in all recovering rats remained microscopically patent for extended periods.

throughout. The only splenectomized rats capable of survival were those whose parasitemias remained low throughout infection, and of these the older the rat the better its chances to survive.

Table 7 shows that splenectomized male and virgin female rats, like intact controls, did not differ significantly in their response to *P. berghei* when ages were identical. Although mature females were found to be smaller than males of the same age, this difference is unavoidable if ages are to be identical. Animals in table 7 are the same as those in the splenectomized group in table 4, category C.

Data on hematological changes in young and adolescent intact and splenectomized rats were collected at 3- to 6-day intervals throughout the course of their infections with *P. berghei*. Hemoglobin values were determined according to Sahli, using 15.6 g = 100% as the basic value, as advocated by Farris and Griffith in *The Rat in Laboratory Investigation*. Data on hemoglobin values and erythrocyte counts are summarized in table 8.

Initial hemoglobin determinations and erythrocyte counts in intact rats revealed the anticipated progressive increase in both values with advancing age (cf. Farris and Griffith, *The Rat*).

As expected, a marked fall in hemoglobin was associated with progressing parasitemia in both splenectomized and intact rats, and this loss was quickly compensated for during latency in surviving animals. Loss of about two-thirds of the hemoglobin was still compatible with recovery, but rats losing more than three-fourths of their hemoglobin succumbed. Intact adolescent rats were able to suppress infection with proportionately less hemoglobin loss than intact young rats.

Splenectomy per se did not affect the hemoglobin content of the blood in young rats, but in the adolescent group it was associated with a slight but significant initial anemia as compared with intact controls.

In all respects erythrocyte counts closely paralleled hemoglobin values throughout the infection. Anemia was therefore due essentially to loss of erythrocytes but not to fundamental changes in their constitution.

Parasitemia peaks were frequently not coincident with observed minimal hemoglobin and erythrocyte values. While the precise correlation of these factors cannot be deduced from our data, since hemoglobin and erythrocyte values were not determined daily, peak parasitemias preceded observed minimal hemoglobin values by 3 to 21 days in 13 cases, or followed them by 3 to 7 days in 3 cases. Peak parasitemias and hemoglobin minima were approximately simultaneous in 10 cases for which there were adequate data. Parasitemias at the time of hemoglobin minima in the non-

TABLE 8.—*Hematological data at varying times during infection of young (A) and adolescent (B) intact and splenectomized rats with* P. berghei. *Figures in parentheses represent numbers of rats in a group.*

Group		Hemoglobin determinations according to Sahli (100%:15.6 g)				Erythrocytes (millions/mm³)			
		before inoculation	minimal in dying rats	minimal in surviving rats	maximum during latency	before inoculation	minimal in dying rats	minimal in surviving rats	maximum during latency
A	Splenectomized	90.6±2.19 (9)	24.4±2.28 (8/9)	*	*	7.82±0.24 (9)	1.69±0.20 (8/9)	3.24 (1/9	*
	Intact	92.9±2.34 (10)	25.4±3.67 (6/10)	34.5±6.46 (4/10)	93.3±6.08 (4/10)	7.54±0.26 (10)	1.61±0.17 (6/10)	2.40±0.43 (4/10)	†
	Standard error of difference between means	3.21	4.32			0.35	0.26		
	P	0.49	0.83			0.45	0.77		
B	Splenectomized	93.7±2.95 (7)	23.0±2.15 (7/7)			8.18±0.20 (7)	1.72±0.28 (7/7)		
	Intact	106.2±2.07 (6)	40.0 (1/6)	68.0±5.11 (5/6)	106.6±3.33 (5/6)	8.52±0.32 (6)	3.96 (1/6)	5.61±0.45 (5/6)	8.98±0.24 (5/6)
	Standard error of difference between means	3.60				0.37			
	P	0.02				0.40			

* Data inadequate.
† Data incomplete, but a sharp rise to the preinoculation level noted in 2 of the 4 rats in this group.

coincident group were frequently very low, and in one case no parasites were observed at a time when hemoglobin was at half the normal level. The fact that peak parasitemia preceded hemoglobin loss in most of the cases suggests the delayed activity of an anemia-inducing factor secreted by living parasites or incorporated in and released from the bodies of parasites destroyed by the host.

DISCUSSION

With advancing age intact rats become less susceptible to *P. berghei*, but this change does not proceed at a uniform rate throughout their development. Up to the age of about 5 weeks parasitemias and mortality rates are high and crisis is delayed until 2 weeks or more after inoculation. Rats 3 months old (immature adults) or older have uniformly low parasitemias and mortality rates, and crisis occurs early, about a week after inoculation. The fact that this fundamental change in response develops rather precipitately during the period of the establishment of sexual maturity suggested that there might be a causal relationship between sexual maturation and age resistance.

This hypothesis was further strengthened by the fact that intact female rats are less susceptible shortly after gestation than virgin females of the same age. Trager (1948) and Trager and McGhee (1950) have shown that egg-laying ducks are markedly less susceptible to *Plasmodium lophurae* than are males or females of the same age with inactive ovaries. Maegraith et al (1952) found that a cow or human milk diet suppresses but does not cure *P. berghei* in mature rats.

Extirpation of functioning rat ovaries, mammae or testes did not, however, support the view that active endocrine function of these glands is intimately connected with susceptibility to *P. berghei*. Females ovariectomized shortly after gestation are as resistant as intact controls with similar histories. Whether this residual resistance is associated with a circulating humoral factor which still remains protective even after its production has been suppressed by ovariectomy was not determined. In the lophurae-infected ducks of Trager and Trager and McGhee a circulating factor was demonstrated by passive transfer. Galliard and Lapierre (1951), following similar reasoning, tested the effect of male hormone (sterandryl) in two sexually immature rats and found that it did not modify *P. berghei* infections.

Table 6 leads to a certain number of conclusions as to the immune mechanisms of the rats in question. The initial state of the infection is interpreted as reflecting the degree of innate immunity. Since plateaus were defined as periods of relative equilibrium between host and parasite lasting for at least a week, infections whose initial phase is a plateau have demonstrable innate immunity. Later stages of the infection (after at least the first week and certainly after crisis) reflect the degree of acquisition of immunity following exposure to the parasite. The development of a crisis following a parasitemia peak is the first unequivocal sign of acquired immunity. However, the suppression of a peak and the maintenance throughout of a low plateau parasitemia is considered to indicate even more efficient acquired immunity than operates in cases where acute peaks occur. Final indication of effective acquired immunity is the ability to survive.

Innate immunity in intact rats increased with advancing age. A high percentage of young intact rats (8/26, or about 30%) had totally inadequate innate immune mechanisms and their fulminating parasitemias rose progres-

sively until death. In 18/26 (about 60%) young and in practically all adolescent and mature intact rats some innate immunity was demonstrable, yielding initial plateau infections.

About half of the young intact rats and nearly all adolescent and mature intact rats developed acquired immunity controlling parasitemia either with or without crisis, as defined above. Although most of these survived, a certain number died in spite of the fact that their parasitemias had been consistently low. Their acquired immunity, capable of controlling parasitemia, was unable to protect them from death.

Innate immunity in splenectomized rats was invariably sufficient to achieve an initial plateau. Since young intact rats frequently had inadequate innate immunity, it is difficult to avoid the conclusion that removal of the spleen from a young rat may favor innate immunity in many cases. No immunological explanation is yet available for the paradox that fulminating parasitemias, so striking a feature of fatal infections in young intact rats, were never observed in splenectomized controls. It is unlikely that this was due to chance since the experimental sample was relatively large.

Plateaus late in infection as indicators of acquired immunity were characteristic of splenectomized rats of all ages but were more likely to be interrupted by acute rises (i.e., by suspension of efficient immunity) in younger than in older rats. In spite of the fact that this demonstrable degree of acquired immunity regularly developed in splenectomized rats, the intact and not the splenectomized state favored ultimate survival. There were survivors in all age groups among intact rats, but, with a single exception, only in the mature group among splenectomized rats. In general, therefore, effective acquired immunity can be produced by extrasplenic tissue only in older rats, whereas the spleen is indispensable to its development in immature rats. Once parasites have invaded more than 20% of its erythrocytes, the splenectomized rat is doomed. This constitutes a fundamental difference between intact and splenectomized rats, since even young intact rats could survive intense parasitemia, whereas even old splenectomized rats were incapable of doing so. The fact, discussed in a previous section, that surviving splenectomized rats were unable to achieve microscopic latency for months after intact controls had done so also points to less effective acquired immunity. Approaching the same phenomenon from a slightly different angle, Black (1951) found that a cured rat, reinfected following splenectomy, maintained patency for as long as 150 days thereafter.

Throughout these experiments the occurrence of plateaus favored but did not presuppose survival. In other words, the ability, innate or acquired, of the intact or splenectomized host to hold the parasite in check even for relatively extended periods did not necessarily presuppose the eventual establishment of effective acquired immunity. The intact rat with a plateau infection was better able to build up an ultimately protective mechanism than was the splenectomized rat with the same type of infection.

Age immunity in rats followed the pattern of resistance increasing with age as in the case of *P. lophurae* in chickens (Trager and McGhee, 1950; Becker, 1950) and not of the reverse age immunity found in ducks infected with the same parasite, in which susceptibility increases with age (Hewitt et al, 1942). Galliard and Lapierre (1951) have observed milder and shorter infections with *P. berghei* in mature than in young

rats. It is of interest that Maegraith and his coworkers (1952), employing a strain of *P. berghei* obtained from the same source as our strain, and also maintained, like ours, for several years by intraperitoneal blood passage, observed a uniformly high mortality rate in mature rats, whereas in our experience the mortality in this age group was very low. This marked difference in susceptibility may be attributable to the fact that they passaged their strain in rats, whereas we maintained ours by mouse passage, thus perhaps inadvertently selecting a strain with reduced virulence for rats although it consistently kills 100% of the recipient mice.

If females were virgin and ages identical, no innate difference was noted in the susceptibility of intact male and female rats to *P. berghei* such as that observed by Bennison and Coatney (1948) between male and female chicks infected with *Plasmodium gallinaceum*.

Although very severe parasitemias were observed in fatal cases of berghei infections in young rats, it seems doubtful whether parasitemia alone is the direct cause of death. Older rats frequently succumbed following very mild parasitemias indeed, particularly after splenectomy. Furthermore, although severest anemias in immature rats sometimes occurred simultaneously with peak parasitemias, as often the two peaks were not coincident; and death seemed more regularly to follow a subminimal anemia of about 1.6 million erythrocytes per mm^3 than any given parasitemia threshold. Galliard and Lapierre also found that death in splenectomized rats more frequently occurred during the recession of parasitemia than at its peak.

It may be that some accumulating parasitic product, apparently more effective or more abundant in splenectomized rats, continues to induce anemia even when parasites have already receded. Such an effect has been observed in *Babesia bigemina* of cattle (Adler, 1953); and, indeed, a widely endorsed hypothetical explanation of the etiology of blackwater fever is based on similar reasoning (Maegraith, 1948). One of us (Zuckerman, 1945), working with *P. gallinaceum* in the chicken, has suggested the possibility that parasites and erythrocytes may share a common antigen. If this were so, the production of antiparasitic antibody might then predispose to erythrolysis and anemia.

Profound differences in the reactions of closely related hosts to the same parasite have frequently been described in studies on host-parasite relationships. Comparison of the results of the present study on splenectomy in rats infected with *P. berghei* with similar data on the field vole (*Microtus guentheri*) infected with the same parasite (Zuckerman and Yoeli, 1951) reveals the fallacy of equating even superficially similar reactions in different hosts. Since intact adult rats have infection courses only slightly more severe than those in intact voles (both characterized by relatively low mortality rates, several weeks' patency, low parasitemias in most cases and benign relapse showers during the first week or two of microscopic latency), one might assume a priori that the underlying immune mechanisms of these two rodents would be fundamentally similar. Splenectomy before inoculation reveals that this is definitely not the case. All voles so splenectomized succumbed following short, intense, fulminating infections, and this was interpreted as indicating that the vole spleen was essential to the functioning of innate immunity. Rodhain (1949) found that the intact cotton rat had an even milder infection than that in intact voles and rats, while its reaction to *P. berghei* after splenectomy

closely paralleled the severe reaction in the splenectomized vole.

Of adult splenectomized rats inoculated with *P. berghei*, only half succumbed, as compared with the 30% mortality rate in intact controls. The 8 survivors and 6 of the 8 rats succumbing all had very mild, plateau-type infections. The innate immunity of the adult rat, in contrast to that of the adult vole, was thus seen to remain virtually undisturbed by splenectomy. Extrasplenic organs of the rat are therefore capable of controlling *P. berghei* during initial phases of infection at a time when such organs in the vole are totally incapable of exercising such control.

The above comparison deals only with the mature rats discussed in the present paper, since the voles were all adult although their exact ages were not on record. Including the younger rat groups in this comparison would have underscored the difference between innate immunity in rats and voles even more strikingly, since in very young rats differences actually favored the splenectomized as compared with the intact animal in certain respects, such as intensity of parasitemia, although some fulminating infections were recorded in this age group.

The fact that splenectomy of very young rats partially suppressed parasitemia on subsequent infection with *P. berghei* seems to run counter to the known role of the reticulo-endothelial system in malarial immunity. Galliard and Lapierre (1951) working with rats of the same young age group also observed this sparing effect of splenectomy on parasitemia, unaccompanied by ultimate protection.

SUMMARY

With advancing age intact rats become progressively less susceptible to *Plasmodium berghei* in that parasitemias become milder, mortality rates fall, and crisis occurs earlier. However, death in fatal cases tends to occur earlier the older the rat. Many young rats dying of infection have fulminating courses, whereas mature rats, whether succumbing or surviving, tend to have initial courses characterized by plateau periods indicating partial suppression of parasitemia. Innate immunity in young, intact rats is therefore inferior to that in adults.

In each age group male and female rats react similarly to *P. berghei* if females are sexually immature or virgin when infected. Although mature females shortly after gestation are less susceptible than mature virgin females, ovariectomy or mammary extirpation in sexually active females or castration in mature males have no perceptible effect on susceptibility of rats to *P. berghei*.

Splenectomy prior to inoculation has an ultimately adverse effect on susceptibility of rats to *P. berghei*, although in certain respects it may temporarily favor host resistance. The adverse effect of splenectomy is expressed in higher mortality rates, particularly in immature rats, in the inability of surviving splenectomized rats to reduce parasitemia to a submicroscopic level (latency), and in the suppression of certain aspects of the age immunity observed in the intact rat, such as accelerated crisis and delayed death.

Temporary increase in host resistance induced by splenectomy may be seen in partial suppression of parasitemia in very young, splenectomized rats, and in the maintenance of protracted plateau periods in splenectomized rats of all ages, even in those eventually succumbing to infection. Immature intact rats succumbing to infection, on the other hand, tend to have fulminating rather than plateau infections. This ability on the part of splenectomized rats to check

the severity of their parasitemias for a time is not associated with protection, since many rats whose parasitemias never rose above a low plateau level eventually succumbed.

While splenectomy of the rat has little effect on innate immunity, it seriously interferes with the establishment and maintenance of acquired immunity to *P. berghei*.

Death is not always coincident with peak parasitemia, but in all cases studied it was associated with maximal anemia, which was of the acute, hemolytic type. The possible mechanism of induction of anemia after the regression of parasitemia is discussed.

REFERENCES

1. Adler, S. 1953, Personal communication.
2. Becker, E. 1950, Iowa Acad. Sc., **57**: 435.
3. Bennison, B. E. and Coatney, G. R. 1948, Science, **107**: 147.
4. Black, R. H. 1951, Ann. Trop. Med. Parasit. **45**:199.
5. Farris, E. J. and Griffith, J. Q., 1949, The Rat in Laboratory Investigation, ed. 2, Philadelphia, London, Montreal, J. B. Lippincott Co.
6. Galliard, H. and Lapierre, J. 1951, Bull. Soc. path. exot. **44**: 185.
7. Hewitt, R., Richardson, A. P. and Seager, L. D. 1942, Am. J. Hyg. **36**: 6.
8. Maegraith, B. 1948, Pathological Processes in Malaria and Blackwater Fever, Oxford, Blackwell Scientific Publications.
9. Maegraith, B., Deegan, T. and Jones, E. S. 1952, Brit. Med. J. Dec. 27, no. 4799, 1382.
10. Rodhain, J. 1949, Ann. Soc. belge de méd. trop. **29**: 483.
11. Trager, W. 1948, J. Parasitol. **34**: 389.
12. Trager, W. and McGhee, R. B. 1950, J. Exper. Med. **91**: 365.
13. Zuckerman, A. 1945, J. Infect. Dis. **77**: 28.
14. Zuckerman, A. and Yoeli, M. 1951, J. Infect. Dis. **89**: 130.

THE COURSE OF INFECTION WITH *PLASMODIUM BERGHEI* IN INBRED CF 1 MICE

IRA SINGER*

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

Although there have been numerous reports on *Plasmodium berghei* infections in mice, only one[1] has dealt with animals of an inbred strain. The present investigation was carried out on white mice of the inbred Carworth Farms #1 (CF 1) strain. For the past two years a consistently reproducible course of infection has been noted in these animals and it has been found that the collected data lend themselves to interpretation according to our present knowledge of malarial infections in general. It is considered worthwhile, therefore, to report on this infection in CF 1 mice in detail.

MATERIALS AND METHODS

The KBG 173 strain of *P. berghei* was obtained from the National Institutes of Health on May 7, 1951, and has been passed biweekly in this laboratory in white mice by the intraperitoneal route. Infected animals have been kept in air-conditioned quarters at about 72 F, with alternating periods of 12 hours of light and 12 hours of darkness.

Five- to six-week-old CF 1 female mice obtained from Carworth Farms were used in all experiments. To initiate infection 12,500,000 parasites were given intravenously. The desired inoculum was obtained by dilution of the donors' blood after red cell and parasite counts had been made. The data reported here have been gathered from several experiments. Expressed as the number of parasites per 10,000 erythrocytes, they represent an average for 60 animals, while an average of 20 to 30 animals was used in describing percentage parasitemia, percentage reticulocytes, and total red blood cells.

Smears for parasite counts, smears for reticulocyte counts, and red cell counts were made daily of blood taken from the tail veins of the animals. Smears for reticulocytes were made over a dried film of 0.3% (in 95% ethyl alcohol) brilliant cresyl blue and were placed in a moist chamber for 5 to 10 minutes. On removal from the moist chamber, they were rapidly dried and examined later the same day. Reticulocytes are expressed as the percentage of red cells present. The smears for parasite counts were dried, fixed in methyl alcohol, and stained with Giemsa. Parasite counts were made according to the method used by Taliaferro and Taliaferro[2] and expressed both as the number of parasites per 10,000 red blood cells and as percentage parasitemia. Due to the presence of many pluriparasitized cells, the number of parasites often exceeded the number of erythrocytes; nonetheless, there were still uninfected red blood cells present.

EXPERIMENTAL

All animals inoculated became infected and died of the infection. The average survival time was 15.23 ± 0.46 days, with a range of 11 to 19 days. No prepatent period was apparent and organisms were always present on the first day of infection. In figure 1 parasitemia is described on a semi-logarithmic scale. The slope of the curve repre-

Received for publication August 26, 1953.

This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

The author wishes to express his appreciation to Dr. Robert M. Lewert and to Dr. William H. Taliaferro for their advice and assistance throughout the course of this work.

* Present address: The Christ Hospital Institute of Medical Research, Cincinnati 19, Ohio.

1. Greenberg, J., Nadel, E. M. and Coatney, G. R. 1953, J. Infect. Dis. **93**: 96–100.

2. Taliaferro, W. H. and Taliaferro, L. G. 1940, J. Infect. Dis. **66**: 153–165.

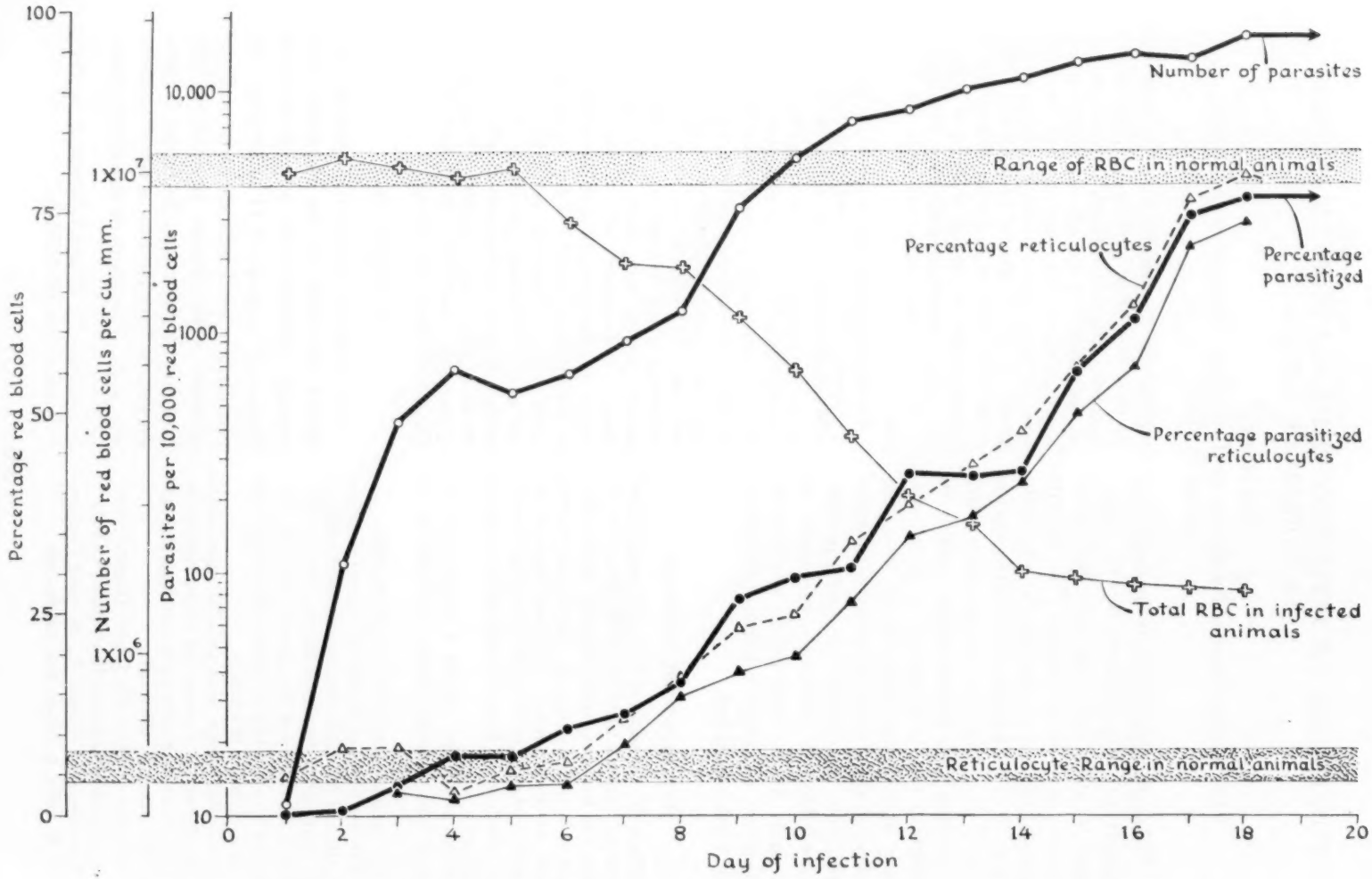


FIG. 1.—The course of infection in CF 1 female mice. Parasites per 10,000 red blood cells, percentage parasitemia, percentage parasitized reticulocytes, percentage total reticulocytes and total red blood cell counts are illustrated.

sents the rate of change in numbers of parasites. It is apparent that during the first 4 days parasites increase at about twice the rate of the 5th through the 10th day. On the 5th day, greater numbers of parasites are killed than are produced and the total count drops. Thereafter, the rate of increase declines until after the 10th day, when it is about one-quarter that of the initial part of the infection. It should be noted here that if parasites are expressed in terms of number per cu mm of blood, they are seen to rise in the terminal stages of the infection despite the severity of the attendant anemia.

Total red cell counts remain essentially normal through the 5th day of infection, when there is a sudden drop in the number of erythrocytes per cu mm of blood. The destruction of red cells continues at a relatively constant rate up to the 14th day of infection, when it tapers off and the total red cell count remains at about 1.4 million red cells per cu mm of blood (fig. 1).

With the progressive increase in numbers of parasites, there is an increase in the proportion of red cells which are reticulocytes. From the 6th day of infection the percentage of reticulocytes is seen to rise well above the normal range. It is paralleled by a rise in the percentages both of red cells which are parasitized reticulocytes and red cells which are parasitized mature erythrocytes. By the 18th day of infection 76% of the red cells are parasitized, 81% of the red cells are reticulocytes, and 73% of the red cells are parasitized reticulocytes; these findings indicate that only 3% of the parasitized erythrocytes are mature cells (fig. 1).

DISCUSSION

Schneider and Montezin,[3] Mercado and Coatney,[4] and Darrow, Gingrich and Prine[5] agree that the parasitemia in mice rises through the 5th day of infection and thereafter proceeds with only slight increases. Darrow et al also call attention to a drop in parasitemia after the 5th day, with subsequent rises. The results cited here are in essential agreement with those of the workers mentioned above. However, it is felt that a different interpretation of these findings is necessary. Mercado and Coatney note that the marked drop in total red cell count coincides with the lowering of the rate of increase of parasites on the 5th day of infection. They suggest that the lack of correlation between the degree of anemia and the parasitemia may be attributed to an increase in the fragility of the infected red cells. It seems that this alone cannot wholly account for the rapid disappearance of erythrocytes and that probably a number of factors are operating to produce the observed results. An examination of figure 1 will show that the drop in total numbers of red cells occurs on the 5th day or very shortly after the time when more parasites have been killed than have been formed. It appears, therefore, that mechanisms of acquired immunity have begun to operate. Unfortunately for the host and the parasite, these humoral and cellular factors never reach a level where the infection can be controlled. The drop in total numbers of parasites on the 5th day represents a suddenly greater ability on the part of the host to kill parasites and to precipitate the occurrence of a minor crisis in the course of infection. With the addition of those humoral and cellular factors of acquired immunity to the relatively ineffectual

3. Schneider, J. and Montezin, G. 1950, Bull. Soc. path. exot. **43**: 144–151.
4. Mercado, T. I. and Coatney, G. R. 1951, J. Parasitol. **37**: 479–482.
5. Darrow, E. M., Gingrich, W. D. and Prine, J. H. 1952, Am. J. Trop. Med. **1**: 927–931.

mechanisms of innate immunity, both the fall in the rate of increase of numbers of parasites and the destruction of red cells may be accounted for. It is interesting to note here that the spleen, liver, and bone marrow exhibit very marked agglutination of parasitized erythrocytes in the sinuses and small blood vessels at this time.[6] It is probable, therefore, that immune mechanisms are causing an agglutination and lysis of red cells, along with an increase in erythrophagocytosis and phagocytosis of parasitized erythrocytes. This would suggest that *P. berghei* evokes essentially the same immune mechanisms but to a lesser degree in the mouse than it does in the rat,[7,8] and that these reactions are similar to the reactions of other hosts to other plasmodia.

Galliard[9] Baldi,[10] Corradetti and Verloni,[11] Hsu and Geiman,[12] and Singer[13] have reported that *P. berghei* shows a marked tropism for immature red blood cells. Figure 1 shows complete agreement with this observation. On the 14th day of infection, 50% of the circulating erythrocytes are immature red cells or reticulocytes. This means that the parasite has an equal opportunity to penetrate either a mature or an immature erythrocyte. At this time 41.2% of the red cells are parasitized reticulocytes, while the total percentage of parasitized red cells is only 42.6. Therefore, out of every 100 cells only 1.4 mature red cells contain parasites, while 41.2 reticulocytes are parasitized. To express this another way, out of every 100 cells parasitized, 96.7 are reticulocytes while only 3.3 are mature red cells. This indicates that the parasite must preferentially parasitize the immature cells.

6. Singer, I. 1953, J. Infect. Dis. **94**: 241–261.
7. Corradetti, A. 1950, Riv. parassit. **11**: 201–209.
8. Fabiani, G. and Vargues, R. 1951, Compt. rend. Acad. d. sci. **233**: 502–504.
9. Galliard, H. 1949, Bull. Soc. path. exot. **42**: 431–433.
10. Baldi, A. 1950, Riv. di malariol. **29**: 349–356.
11. Corradetti, A. and Verloni, F. 1951, Riv. Parassit. **12**: 69–84.
12. Hsu, D. Y. M. and Geiman, Q. M. 1952, Am. J. Trop. Med. **1**: 747–760.
13. Singer, I. 1953, J. Infect. Dis. **92**: 97–104.

SUMMARY

Blood-induced infection of white mice with *Plasmodium berghei* is characterized by a rapidly increasing parasitemia for the first 4 days, followed by a drop in numbers on the 5th day (minor crisis), and by a less rapidly increasing parasitemia for the remainder of the infection. The rise in numbers of parasites is accompanied by a rise in the percentage of reticulocytes in the circulating blood and the development of a severe anemia by the host. The course of infection is interpreted in the light of possible mechanisms of both innate and acquired immunity and the parasites' preferential parasitism of immature red blood cells.

THE CELLULAR REACTIONS TO INFECTIONS WITH *PLASMODIUM BERGHEI* IN THE WHITE MOUSE

IRA SINGER*

From the Department of Bacteriology and Parasitology, The University of Chicago, Chicago 37, Illinois

Melanosis and lipoidosis of the spleens and livers of mice infected with *Plasmodium berghei* have been described by Levaditi and Vaisman[1] and the intensity of these processes were related to the degree of parasitemia. Later, Galliard and Lapiere[2] reported the occurrence of proliferative activity in the young myeloid elements of the bone marrow during the course of this infection. Certain investigators[3] have claimed that exoerythrocytic stages of the parasite may be found in the histiocytes of the liver and bone marrow and very rarely in the capillary endothelium. Others[4] have denied their presence. The present study is a description of the progressive pathology and the cellular reactions in mice with *P. berghei* infections. At no time during the course of this work were exoerythrocytic forms of the parasite seen. Where reactions to this infection differ greatly from the responses of other animals to other plasmodia, they will be contrasted; but no attempt will be made to describe the extensive literature in this area, for that has been very adequately dealt with elsewhere.[5–7]

METHODS

Forty 5- to 6-week-old CF 1 mice (20 female and 20 male) were infected intravenously with 12,500,000 parasites. One male and one female were sacrificed at 24-hour intervals for 16 days. The animals were etherized and bled to death from the brachial artery. Various tissues were removed and fixed, for a period of 18 to 24 hours, in Zenker-formol (19 parts of Zenker's solution to 1 part of neutral formalin). After embedding in celloidin, sections were cut 8 μ in thickness and mounted serially. All sections were stained with

Received for publication August 26, 1953.

This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service and by grants from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

The author wishes to express appreciation to Dr. William H. Taliaferro and to Dr. Robert M. Lewert for their advice and assistance throughout the course of this work.

* Present address: The Christ Hospital Institute of Medical Research, Cincinnati 19, Ohio.

1. Levaditi, C. and Vaisman, A. 1950, Histopathologie de l'infection provoquée chez le souris par le *Plasmodium berghei* (1) le rat, réservoir de virus. Bull. Soc. path. exot. **43:** 693–696.
2. Galliard, H. and Lapierre, J. 1950, Modifications hematologiques au cours de l'infection a *Plasmodium berghei* chez le souris. Essais de diverses substances non spécifiques. Bull. Soc. path. exot. **43:** 317–323.
3. Van den Berghe, L., Vincke, L. and Chardonne, M. 1950, La phase tissulaire de *Plasmodium berghei*. Ann. Soc. belge de méd. trop. **30:** 79–82.
4. Galliard, H. and Lapierre, J. 1950, Recherches sur le developpement initial de *Plasmodium berghei*. Bull. Soc. path. exot. **43:** 410–412.
5. Taliaferro, W. H. and Cannon, P. R. 1936, The cellular reactions during primary infections and superinfections of *Plasmodium brasilianum* in Panamanian monkeys. J. Infect. Dis. **59:** 72–125.
6. Taliaferro, W. H. and Mulligan, H. W. 1937, The Histopathology of Malaria with Special Reference to the Function and Origin of the Macrophages in Defense, Indian Medical Research Memoir, no. 29, Calcutta, Thacker, Spink Co., Ltd.
7. Maegraith, B. 1948, Pathological Processes in Malaria and Blackwater Fever, Springfield, Charles C Thomas.

hematoxylin-eosin-azur II, according to the method of Maximow.[8] In addition to being the connective tissue stain of choice, HEA proved to be excellent for the recognition of malarial parasites in erythrocytes in the tissues, even though it did not afford sharp differentiation of the parasite nucleus from the cytoplasm.

Sections were made of the spleen, liver, bone marrow, lung, heart, kidney, adrenal, thymus, tongue, diaphragm, stomach, small and large intestine, and the axillary, inguinal, lumbar, and mesenteric lymph nodes. These organs will be described in the order of the severity of their response to the infection.

EXPERIMENTAL

Spleen

In the normal animal.—The white pulp of the normal mouse spleen is made up of lymphatic tissue similar to that found in the cortex of lymph nodes. It forms a more or less continuous sheath around the branches of the arterial system and contains typical germinal centers (secondary nodules) eccentrically placed with respect to the arterioles.[9] A marginal zone of medium and small lymphocytes surrounds the germinal centers. The marginal zone is ringed by an area of reticular cells which serve as the inner boundary of the transitional zone. Snook[10] describes these reticular cells as being flattened and continuous with the endothelium of the nodular capillaries. They appear to form a series of anastomosing cleft-like spaces which he refers to as the perifollicular spaces. Examination of plate III and plate II, fig. 1, shows that these cells closely resemble the littoral cells of the venous sinuses, and may indicate that the spaces formed by them are actually venous sinuses. Varying greatly in size, and gradually merging with the red pulp, the transitional zone is populated almost exclusively by medium lymphocytes.

The red pulp occupies about one-third the volume of the spleen. Small concentrations of erythropoietic tissue may be found throughout the red pulp but are especially noticeable immediately adjacent to the capsule and the trabeculae. These small groups of 10 to 50 deeply stained basophil cells contain chiefly basophil erythroblasts, some polychromatophil erythroblasts, hemocytoblasts and a few normoblasts. Granulocytopoietic cells are somewhat scattered throughout the organ but appear to prefer areas close to the capsule and trabeculae. Plasma cells, reticular cells, and a few erythrocytes may also be found in the splenic cords. The splenic cords fill the spaces between the terminal venous sinuses. According to Knisely,[11] some of the arterial capillaries connect directly with the venules while others, after a short course, connect with the afferent ends of the venous sinuses. He further believes that the sinuses may intercommunicate to form multiple sinus routes or may form a single route, following which they open into the venules. Erythrocytes are able to leave or enter the closed vascular system only by the individual penetration of its walls; and it is only in traumatized spleens that rapid changes permit the passage of large numbers of erythrocytes into the red pulp.

In the infected animal.—The spleens of mice infected with *P. berghei* are

8. Maximow, A. A. 1909, Über zweckmässige Methoden für cytologische und histogenetische Untersuchungen am Wirbeltierembryo mit spezieller Berücksichtigung der Celloidininschnittserien. Ztschr. f. wissensch. Mikr. **26**: 177–183.
9. Bloom, W. 1948, Histopathology of Irradiation from External and Internal Sources, New York, McGraw-Hill Book Co., Inc.
10. Snook, T. 1950, A comparative study of the vascular arrangements in mammalian spleens. Am. J. Anat. **87**: 31–77.
11. Knisely, M. H. 1936, Spleen Studies. I. Microscopic observations of the circulatory system of living unstimulated mammalian spleens. Anat. Rec. **65**: 23–50.

rapidly changing organs. Grossly, the organ is enlarged by the 2nd day of infection and has changed from bright red to dark maroon in color, the usually mottled appearance having already disappeared. From the 2nd day, the coloration deepens and the enlargement increases until the 7th day, when deepest color and maximum size have been attained.

Along with the changes in size and color, there is a marked transformation in the architecture of the organ. As illustrated in plate I, this transformation is complete by the 7th day of infection. Cross sections of spleens removed from animals sacrificed during the course of infection are shown. The primary increase in white and red pulp, and the subsequent depletion of the white pulp are readily seen. In addition, the rapid increase in the size of the organ is evident from observation of plate I.

Hyperplasia of the white pulp is apparent on the 1st day of infection and continues through the 4th day. Afterwards, recession starts; and by the 7th day of infection, severe depletion which is maintained throughout the infection is evident. Terminally discrete splenic nodules are seen. These seem to be bare germinal centers separated from the red pulp only by the reticular cell boundary of the original transitional zone (plate II, fig. 1). The transitional zone itself has virtually disappeared; there are neither small nor medium lymphocytes present. The predominating cells in the secondary nodule are large lymphocytes. The largest of these (plate II, fig. 1 and plate III, large lymphocyte G) giant basophilic lymphoid cells resemble large lymphocytes but are half again as large and usually appear when the white pulp shows marked reactivity to infection. Taliaferro[6] called these giant large lymphocytes and in infections with *Plasmodium gallinaceum*[12] believes they have the power to develop into several types of connective tissue cells. He has identified[13] this cell with the one which has variously been called an acute splenic tumor cell, a myeloblast, a "lymphoblastic" plasma cell, a developing or immature plasma cell, a macrohistiocyte, and a basophil macrophage. Accompanying the hyperplasia of lymphatic tissue is a large amount of lymphorrhexis, which increases as the infection progresses. Cellular debris resulting from this process may be found in great quantities in large macrophages (plate II, fig. 1).

Concomitant with the primary hyperplasia of the white pulp is a severe hyperemia. The spleen is congested on the 1st day of infection and by the 2nd is tightly packed with red cells. Congestion remains evident through the 6th day of infection, after which it decreases in severity (plate I). During this period, the transitional zone of the white pulp is often obscured by red blood cells.

With the developing infection, erythropoiesis progressively increases. The cells of this series gradually replace the hyperemic areas of the spleen until they, rather than red cells, ring the lymphatic tissues. After the 7th day of infection, the spleen is chiefly engaged in erythropoiesis. It occurs everywhere in the organ except in what remains of the white pulp and in the blood vessels. As the infection progresses, the number of mitotic figures and the amount of cellular debris increase accordingly; and giant large lymphocytes (plate III) make their appearance. In the 1-day-old infection, less than 1 mitosis per oil immersion field can be seen. On the 9th day, 5 to 10 can be found; and by the

12. Taliaferro, W. H. 1953, Unpublished work.
13. Taliaferro, W. H. 1949, The cellular basis of immunity. Ann. Rev. Microbiol. **3**: 159–194.

16th day, almost 1 cell in every 5 is in the process of mitosis (plate VII, fig. 5). It should be noted here that the polychromatophil erythroblasts of the mouse do not show the typical checkerboard arrangement of chromatin in the nucleus that has been described for other animals[14] (plate III and plate II, fig. 1).

Myelopoiesis does not appear to increase appreciably during the course of infection and remains diffuse and scattered throughout the spleen.

Parasitized erythrocytes can be found on the 1st day of infection. They are scarce and scattered and remain so until the 4th day. At this time they appear to be aggregated in the venous sinuses and small blood vessels. Most of the parasites are at the segmenter stage of development. The clumping or agglutination of parasitized erythrocytes is evident through the remainder of the course of infection (plate III).

Manifestations of the mobilization of the forces of innate immunity become evident on the 1st day of infection. Tissue macrophages are seen containing malarial pigment. The amount of phagocytosis increases progressively with the infection. From the 2nd day, pigment and active macrophages are most evident in the areas immediately surrounding the splenic nodules, i.e., the transitional zone. Apparently the infected erythrocytes are emptied into the perifollicular spaces along with arterial blood from the capillaries which course through the splenic nodules. Some of these infected cells pass between the littoral cells (the reticular cells surrounding the marginal zone of the splenic nodules); and before penetrating very deeply into the red pulp, they are ingested by tissue macrophages Starting with the 4th day of infection, medium-sized phagocytic polyblasts are seen containing ingested pigment. Except for the spleen of the animal sacrificed on the 16th day of infection, these cells are always found in, or in close association with, the small blood vessels of the organ; on the 16th day they are seen in all parts of the red pulp. In the early stages of the infection the pigment is a powdery, diffuse substance; while in the later stages, it is coarse and clumped within the macrophages (plate VI, fig. 2). This is probably due to progressive phagocytosis and collection of pigment by these cells.

14. Maximow, A. A. and Bloom, W. 1952, Textbook of Histology, ed. 6, Philadelphia and London, W. B. Saunders Company, p. 84.

The cross sections of spleens taken from mice on various days of infection show changes in size and architecture. ×14.

FIG. 1.—From an uninfected mouse.

FIG. 2.—From a mouse with a 1-day-old infection. Hyperplasia is evident in both white and red pulp. The clear areas surrounding the white pulp are hyperemic.

FIG. 3.—From a mouse with a 3-day-old infection. There is continuing hyperplasia of both white and red pulp and hyperemia has increased in severity.

FIG. 4.—From a mouse with a 4-day-old infection. Hyperplasia of the white pulp has reached its peak and erythropoiesis continues. Hyperemia is beginning to recede.

FIG. 5.—From a mouse with a 6-day-old infection. The area taken up by the white pulp has begun to decrease and is being replaced by the proliferating erythroid tissue of the red pulp.

FIG. 6.—From a mouse with a 9-day-old infection.

FIG. 7.—From a mouse with a 14-day-old infection. The spleen of this animal is almost identical in size and architectural appearance with that of the mouse sacrificed on the 9th day (fig. 6). The spleen has become almost wholly erythropoietic and all that remains are the pale staining, discrete, bare germinal centers.

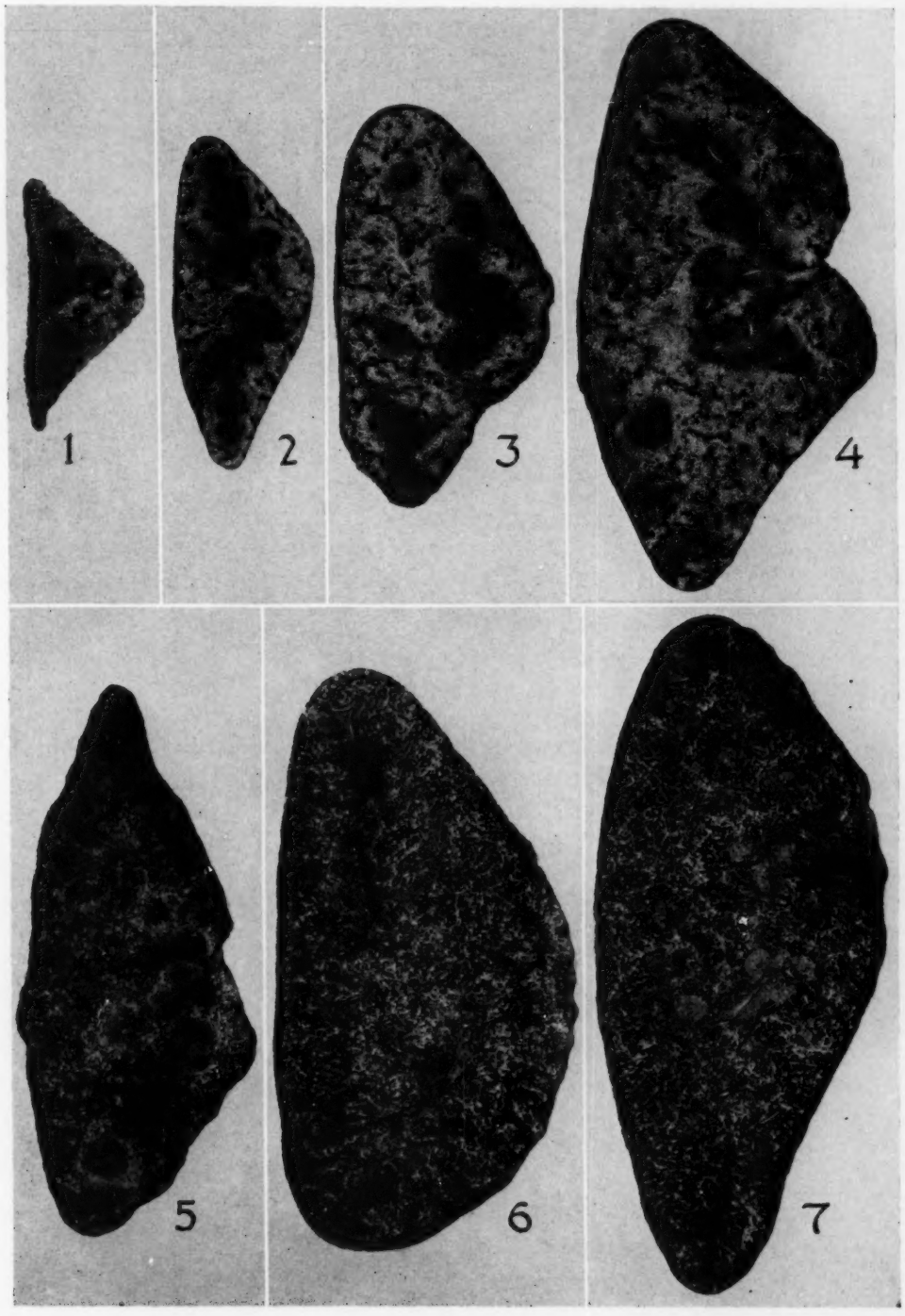


PLATE I

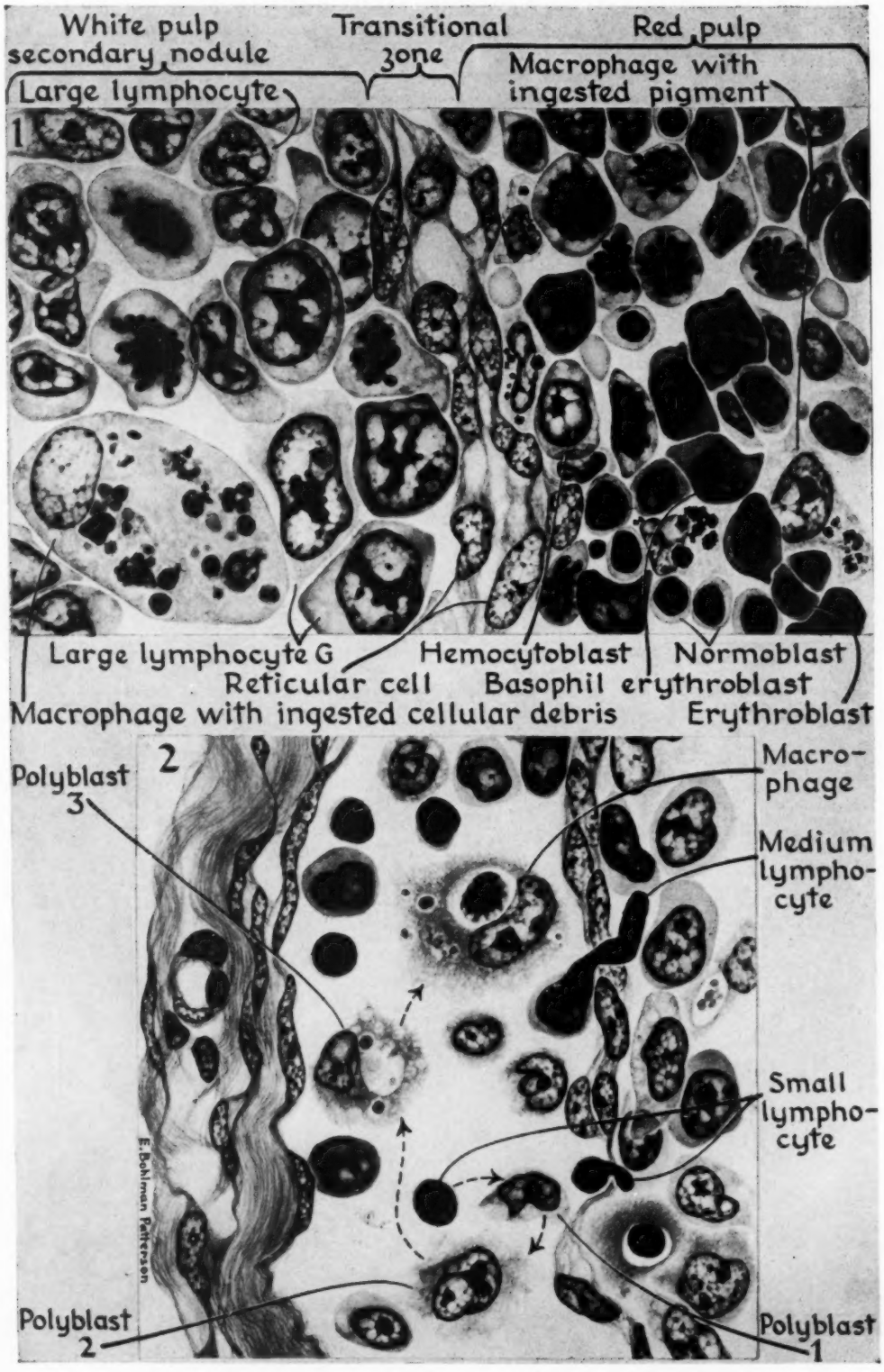

White pulp
secondary nodule
Large lymphocyte
Transitional
zone
Red pulp
Macrophage with
ingested pigment
1
Large lymphocyte G
Reticular cell
Hemocytoblast
Basophil erythroblast
Normoblast
Erythroblast
Macrophage with ingested cellular debris
Polyblast
3
2
Macro-
phage
Medium
lympho-
cyte
Small
lympho-
cyte
E. Behrman Patterson
Polyblast
2
Polyblast
1

PLATE II

Liver

In the normal animal.—Livers of normal mice resemble those of other mammals. The parenchymal cells vary in their staining properties and may often be binucleate. The nuclei are large with a coarsely granular membrane and contain from 4 to 8 nucleoli. The cytoplasm is foamy with stained portions which appear finely granular. The supporting framework or capsule of Glisson is poorly developed and divides the liver into indistinct lobules. Cellular infiltration is often seen immediately about the interlobular vessels. These infiltrates usually contain a few heterophils, some eosinophils, and numerous mononuclear cells. The littoral cells of the sinusoids are flat and deeply stained.

In the infected animal.—The livers of mice infected with *P. berghei* show somewhat marked but inconsistent changes. Grossly, the liver is slightly enlarged by the 3rd day of infection. It continues to enlarge until the 7th, when it is extremely friable and easily torn. During this period the color changes from a pink tan to a dark brown; and by the 16th day of infection, the organ has become almost black.

Few, apparently mild, degenerative changes occur during the course of infection. These changes vary from day to day and probably reflect the variance in the individual animal's power to combat this acute infection.

At no time is congestion particularly severe. On the 2nd day of infection both the portal and hepatic vessels are filled with red cells, and a few of the sinusoids are congested. By the 3rd day, only the sinusoids contain erythrocytes. This condition persists through the infection; but the character of the congesting materials changes to parasitized erythrocytes, phagocytes, and infiltrating cells.

Focal necrosis of liver parenchyma occurs rather inconsistently. It usually involves only 1 or 2 cells and is not confined to any particular area of the lobule. On the 5th, 8th, and after the 12th days of infection, dilatation of the sinusoids is evident; and concomitantly, there is marked condensation of liver cell cytoplasm. This phenomenon appears to be due to pressure atrophy but is not followed by the expected generalized necrosis. Condensation progressively increases in severity until the cells appear greatly distorted on the 15th day. Cytoplasmic condensation in liver cells may well represent a depletion of fat, glycogen, and other storage materials from the cells which cannot be replaced due to the infection.

The forces of innate immunity come into play on the 1st day of infection. Some of the Kupffer cells contain ingested pigment; and most of the littoral cells appear swollen and do not stain as densely as those of normal animals. By the 2nd day, numerous phagocytic cells are seen in the sinusoids. Hypertrophy of the Kupffer cells has occurred. These large, often triangular, cells have nuclei which vary from the darkly stained type seen in the normal liver to a pale vesicular type reminiscent

FIG. 1.—Section of the spleen of a mouse killed on the 16th day of infection, showing a portion of the splenic nodule (left) separated only by reticular cells of the transitional zone (middle) from the red pulp (right). Note the increase in number and the mitotic activity of the larger lymphoid cells. ×1900.

FIG. 2.—The cortical sinus of a mesenteric lymph node of a mouse sacrificed on the 15th day of infection showing the histogenesis of macrophages within the sinus. Note the hypertrophy of lymphocyte to polyblast to macrophage. ×1900.

of the tissue macrophages of the spleen. Medium polyblasts are apparent by the 4th day of infection. Phagocytosis progressively continues until the 16th day, when it suddenly spurts in intensity and the ingested pigment is so heavily clumped within the macrophages that it obscures their nuclei (plate VI, fig. 1). During the course of infection cells containing malarial pigment are occasionally seen in mitosis.

The most striking and consistent change seen in the livers of infected mice is a progressive cellular infiltration of the interlobular areas. Although there is some mantling in the periportal areas of normal livers by granulocytes and mononuclear cells, this does not occur to any appreciable extent and can be easily contrasted with infected livers. With the 1st day of infection an increased concentration of cells, which progressively grows larger as the infection proceeds, is seen about the interlobular ducts. Early in the infection, the cells are chiefly leukocytes and hypertrophying lymphocytes (plate VII, fig. 1). By the 8th day, erythropoietic cells have also appeared in these areas. From that time on, erythropoietic cells increase until, late in the infection, they outnumber lymphoid cells (plate VII, fig. 2).

By the 5th day of infection, megakaryocytes appear in the sinusoids and increase in number as the disease progresses. There is an attendant increase in erythropoiesis. As early as the 1st day of infection, hypertrophying mononuclear cells are evident in the sinusoids and persist in this location throughout the course of infection, forming nests of cells which increase in size and numbers. The histogenesis of macrophages from these cells can be followed. By the 5th day, a few groups of erythropoietic cells which are made up primarily of basophil erythroblasts are evident. As the infection proceeds, more of these erythropoietic nests become apparent. Usually they are composed of one type of cell. Terminally, cells of the erythropoietic series are far more prevalent in the sinusoids than are those of the lymphoid series. It was noted in the spleen that severe white pulp depletion was evident by the 7th day. It is interesting to remark here that after this time there is no great increase in the quantity of round cell infiltration of the liver sinusoids. Proportionately, the amount of erythropoietic tissue increases at a much greater rate; this too, is true of the spleen. It seems probable that the liver is merely reflecting changes in the spleen and that the proliferative elements of the blood found in the sinusoids have been carried there in the blood vessels from the spleen.

Parasitized erythrocytes are found in the liver on the 1st day and throughout the course of infection. They are often seen packing the sinusoids, in macrophages, and in the blood vessels.

Bone marrow

In the normal animal.—At the time of sacrifice, femurs were removed from all animals, split, fixed and embedded in the usual manner, decalcified, and then sectioned. Hemopoietic cells fill the shaft of the bone with blood sinuses occupying the intervening spaces. Cells of one type can often be found in groups, e.g., 10 to 20 erythroblasts may appear in one area. Approximately one-third to one-half of the hemopoietic cells in the marrow are erythropoietic. Of these, about one-third to one-half are normoblasts; the remainder are in earlier stages of development. The other cells filling the meshwork of the marrow belong to the granulocyte series. Many of these are mature. There are usually 5 to 10 eosinophils per oil immersion field. The heterophils are difficult to distin-

guish in their earlier stages of development. They stain palely and the granules do not stain at all. At least half of the granulocyte series is at the myelocyte stage of development, often with doughnut-shaped nuclei. Scattered throughout the marrow, there is a small number of lymphocytes and plasma cells, and about 8 megakaryocytes per microscopic field (300 μ radius). Hemocytoblasts are uncommon and mitotic figures are difficult to find.

In the infected animal.—Although the changes occurring in the bone marrow of animals infected with *P. berghei* are not spectacular, they are nonetheless marked. These changes are difficult to assay. Quantitative counts cannot be made because the cells usually appear in groups of one type, of varying size, and at irregular intervals. The description which follows represents for the most part a general impression of the changes which occur during the course of infection.

The over-all reaction of the bone marrow is a generalized hyperplasia of all the myeloid elements, with changes in the ratio of erythropoietic to granulocytopoietic cells. On the 2nd day of infection, the erythropoietic elements of the bone marrow have increased. They comprise well over 50% of the cells in the reticular meshwork and continue to increase slightly through the remainder of the infection. At first the normoblast predominates; but later, earlier erythroblasts are more easily found. By the 4th day of infection, there is about 1 mitosis per 2 oil immersion fields. On the 12th day, there is 1 per oil immersion field; and by the 16th day, 10 to 20 mitotic figures can be found in each field.

In the early stages of the infection there is a slight increase in numbers of megakaryocytes. This is maintained until the 12th day, when the number drops to one-half the previous level and remains at this low level until the infection has run its course.

From the 2nd day of infection, parasitized erythrocytes are seen in the marrow. Only rarely do they appear in the tissue reticulum. Usually, infected cells are seen in the sinuses and vessels where they have aggregated and often appear to be agglutinated or sticking to the vessel walls.

Macrophages become evident on the 6th day of infection. They are mainly confined to the sinuses and blood vessels and are only rarely seen in the stroma of the marrow. Since few of the parasitized erythrocytes reach the marrow parenchyma, reticular cells with phagocytic potential have no opportunity to act. During the terminal part of the infection macrophages are seen in the marrow. These, though, appear to be of hematogenous origin and have probably wandered out of blood vessels carrying pigment with them (plate VI, fig. 3).

Lymph nodes

In the normal animals.—The normal node is characterized by a general lymphopoiesis. Although the nodules are the most active areas involved in this process, large lymphocytes, mitotic figures, and cellular debris may be found throughout the cortex and medulla (especially the cords). The presence of eosinophils is a constant characteristic of the normal nodes. In the cortex, they are scattered and are usually mature. In the medullary cords, they are found in groups and in various stages of development, indicating that a small amount of eosinophilopoiesis occurs in normal lymph nodes.

In the infected animal.—At necropsy samples of axillary, inguinal, lumbar, and mesenteric lymph nodes were taken. No gross changes in any of these were noted at the time. Microscopically, the

reaction to infection is essentially the same in all the nodes. The mesenteric nodes appear slightly more reactive than the others. During the early stages of infection, there is an inflammatory-like response which is restricted to the medullary cords. The cords become swollen and packed with cells, and seem to obliterate the sinuses. At least half of these cells are granulocytes and are seen in all stages of development. At times eosinophils predominate, while at other times heterophils predominate. This inflammatory-like response is transitory and recedes by the 8th day of infection, although some granulocytopoiesis does persist.

On the 12th day of infection, megakaryocytes can be found in the inguinal and mesenteric nodes. Histogenesis of megakaryocytes occurs in the cortical sinuses of these nodes and is illustrated in plate IV, figures 1 to 4. It appears that they are developing from both reticular cells and large lymphocytes.

A generalized hyperplasia of lymphatic tissue becomes evident on the 6th day of infection and increases progressively during the course of infection. The number of large lymphocytes and of mitotic figures in both lymphocytes and reticular cells increases; and greater quantities of nuclear debris are present. Giant large lymphocytes appear in the germinal centers. Concomitant with the enhanced activity in the nodules, a greater amount of lymphopoiesis is seen in the cortex and medulla. Lymphorrhexis and dividing cells become more evident in these areas. A depletion of small lymphocytes occurs with the hyperplastic activity in the nodules. The coronas of small lymphocytes surrounding the lymphatic nodules become narrow and are not as dense as those in normal animals and in nodes removed earlier in the infection. Figures 1 to 4 in plate V contrast the changes in a nodule in the inguinal lymph node of an infected animal with the nodule of an uninfected mouse. Transport of lymphocytes is evident and sinuses are often seen packed with small lymphocytes which are moving away from the cortex.

Macrophages with ingested malarial pigment can be found from the 10th day of infection. These cells appear to be of hematogenous origin and may be found in both the cortex and medulla. They are always close to a blood vessel and have probably wandered out carrying the pigment with them.

Along with the changes described in the nodes is a progressive depletion of fat from the surrounding adipose tissue. On the 16th day no fat globules can be found; and the fatty connective tissue has simply become a highly vascular mass of cells.

Lungs

In the normal animal.—The interalveolar capillaries are filled chiefly with red cells and varying numbers of lymphocytes and granulocytes. Occasionally a dust cell is seen.

In the infected animal.—During the first 4 days of infection there is a slight increase in the number of granulocytes and lymphocytes seen in the interalveolar capillaries. From the 5th through the 7th day a rare macrophage can be found; lymphocytes and heterophils are seen to increase in number; a few lymphocytes are in mitosis and many of the heterophils appear immature. On the 8th day changes are marked and a tremendous increase in the amount of phagocytosis occurs. All of the phagocytes are in the capillaries. Macrophages in all stages of development are present, ranging from lymphocyte or monocyte to polyblast to the full grown cell with its pale large nucleus. Rarely, plasma cells and polychromatophil erythroblasts are seen in the capillary vessels.

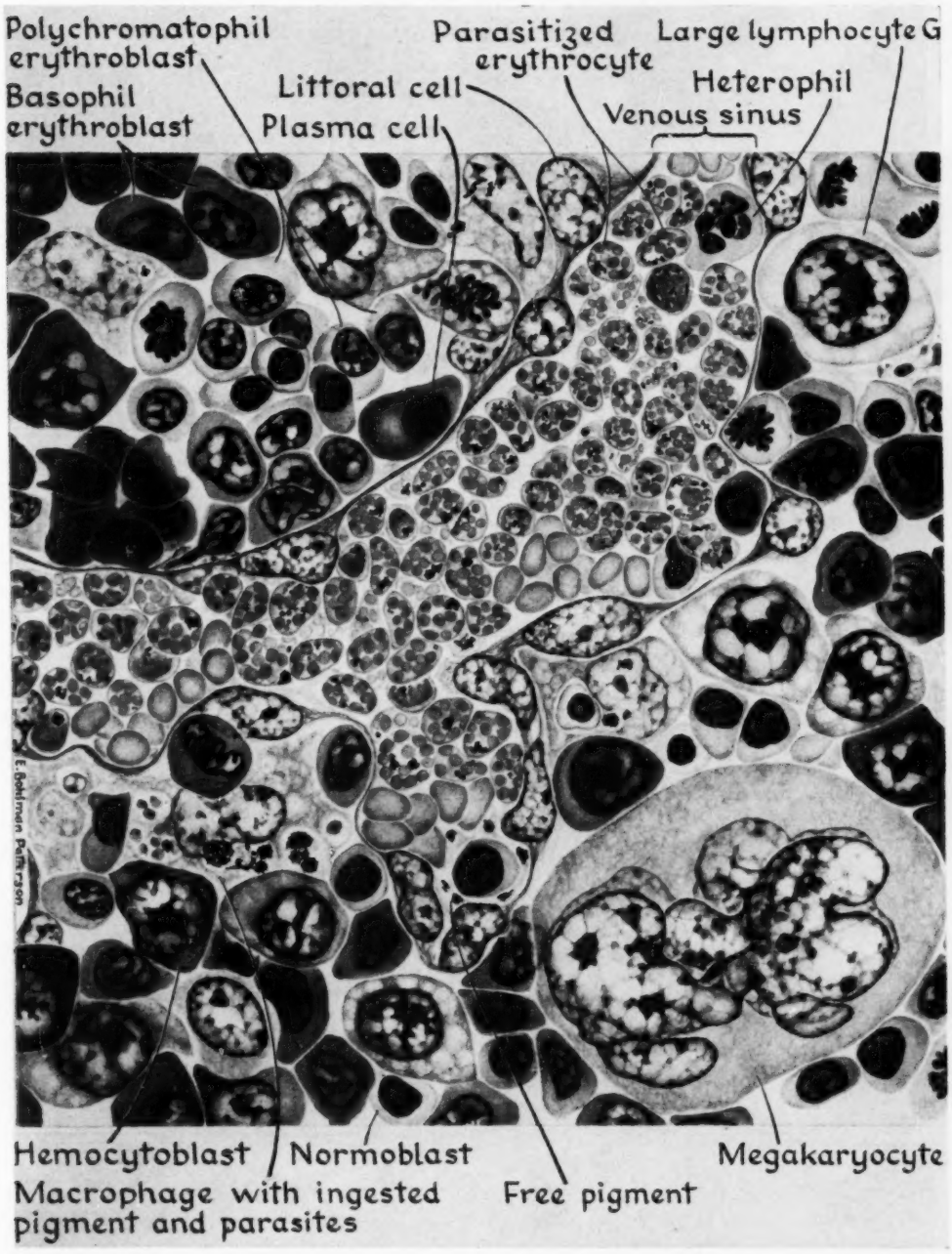


PLATE III

A portion of a venous sinus and splenic cord of the spleen of an animal sacrificed on the 9th day of infection, showing the packing of parasitized erythrocytes into the venous sinus. Note that most of the parasites are in the segmenter stage of development and that mature red cells are rare in the splenic cords. There is a large amount of erythropoietic activity. The cells labeled large lymphocyte G are the same as those shown in plate II, fig. 1, and are rarely found in normal animals. ×1800.

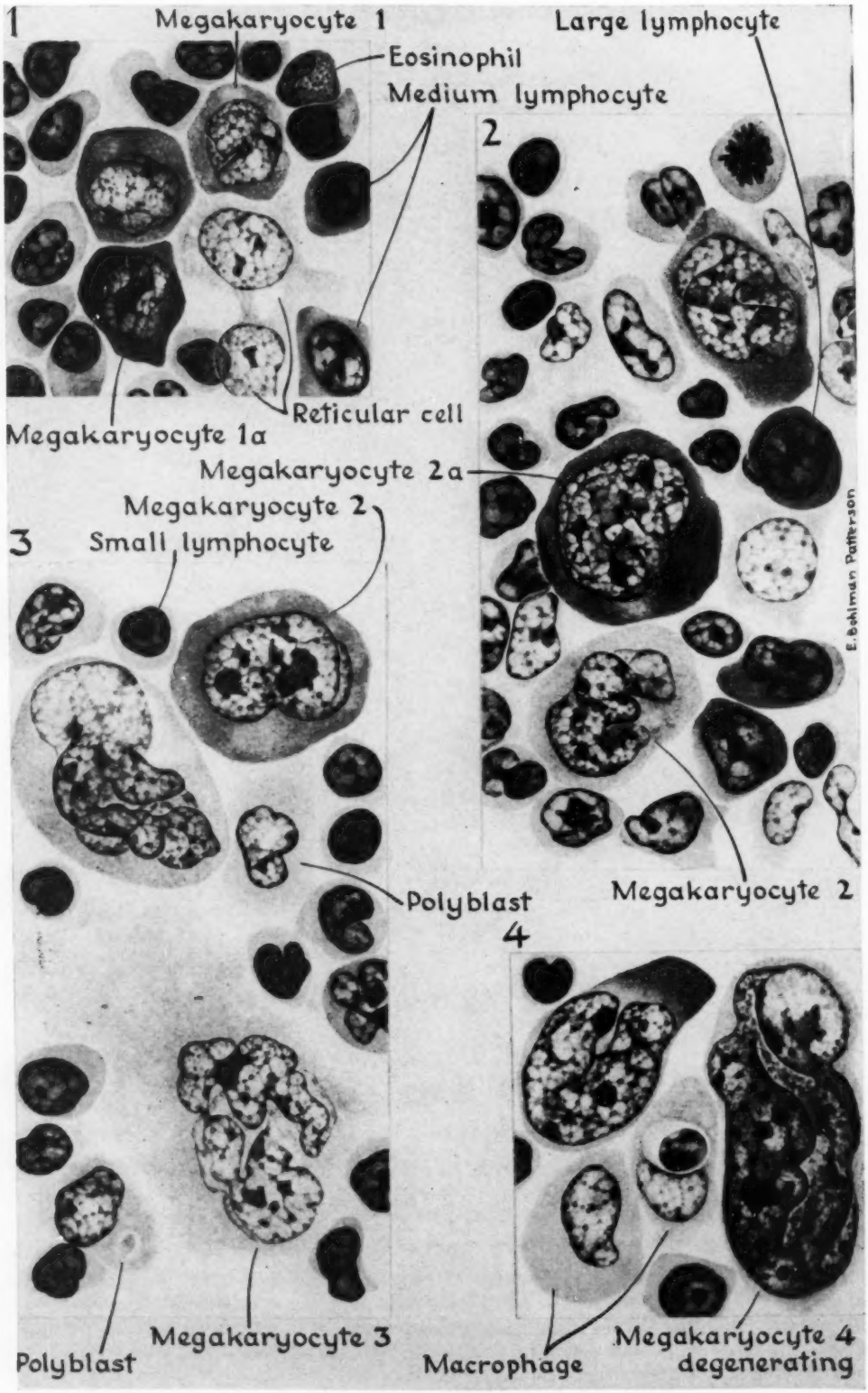

1
Megakaryocyte 1
Eosinophil
Medium lymphocyte
Reticular cell
Megakaryocyte 1a
2
Large lymphocyte
Megakaryocyte 2a
Megakaryocyte 2
3
Small lymphocyte
Polyblast
Megakaryocyte 2
4
Polyblast
Megakaryocyte 3
Macrophage
Megakaryocyte 4
degenerating
E. Bohlman Patterson

PLATE IV

Pigment within the macrophages is usually in small clumps or of powdery appearance (plate VI, fig. 4). Heterophils containing ingested pigment could not be found. Parasitized erythrocytes are evident in the capillaries throughout the infection.

Adrenals

A progressive and remarkable change seen in the adrenals of infected mice is the loss of lipid from the cells of the fascicular zone. Apparent by the 8th day of infection, it is shown by changes in the cell structure. The cells lose their spongy appearance; the cytoplasm condenses, has a ground glass appearance, and stains more deeply eosinophilic (plate VII, fig. 3 and 4).

As early as the 2nd day of infection, parasitized erythrocytes are seen in the sinusoids; and by the 7th day, macrophages are also present. The intensity of phagocytosis increases slightly during the course of infection but at no time is it extensive. All of the macrophages are of hematogenous origin; there is no evidence of phagocytic activity by the sinuosoidal endothelium (littoral cells).

The adrenals of mice sacrificed on the 9th and 12th days of infection show some noteworthy changes. In the former a small amount of extramedullary lymphopoiesis is evident in an area immediately adjacent to the capsule. It contains medium and small lymphocytes. In the latter, small areas of myelo- and erythropoiesis are seen in the medullary sinusoids. Various developmental stages of both granulocytes and red cells are evident.

Kidney

No gross pathological changes were seen in the kidneys of infected mice. Except for swelling of the glomerular epithelium after the 6th day of infection, both the glomeruli and tubules remain essentially normal.

Parasitized erythrocytes are seen in the larger vessels on the 1st day of infection. On the 2nd, they are in the intertubular capillaries, but rarely in the vessels of the glomeruli. From the 7th day, the vessels often appear to be occluded by both infected and uninfected red cells.

Phagocytic activity in the kidney is not remarkable. Macrophages with ingested pigment are rarely seen in the intertubular connective tissue and only occasionally in the intertubular and glomerular capillaries. Judging from the staining of the nuclear chromatin, these cells appear to be of hematogenous origin.

On the 8th day there appears to be some hypertrophy of reticular cells of the intertubular connective tissue. A few are seen to become more dense and take a deeper basophilic stain. Often they resemble hemocytoblasts and strongly suggest that the reticular cells are exerting their mesenchymal potencies. There is also some cellular infiltration of these areas. Groups of mononuclear cells can be found. These are made up chiefly of large and medium lymphocytes along with a few small lymphocytes (plate VII, fig. 6). Rare mitotic figures can be found among these cells.

←«

The histogenesis of megakaryocytes in a cortical sinus of a mesenteric lymph node of a mouse sacrificed on the 12th day of infection. Megakaryocytes appear to originate from either large lymphocytes or reticular cells. Two young megakaryocytes are shown in figure 1. The one marked 1a has definite lymphoid properties, showing a basophilic cytoplasm and a lymphocyte type of nucleus. The one marked 1 seems to have arisen from a reticular cell. Figures 2, 3, and 4 show a graded series of megakaryocytes. Those marked with an a have lymphoid characteristics. Megakaryocyte 4 seems to be degenerating. ×2000.

Thymus

Up to the 11th day of infection the thymus remains essentially mormal. At this time involution occurs. The deeply basophil thymocytes disappear progressively until there is no clear distinction between cortical and medullary areas. There is a progressive accumulation of cellular debris in the gland, some of which has been ingested by macrophages. Granulocytopoiesis, especially of eosinophils, occurs to a small extent. Occasionally macrophages with ingested pigment are seen, but only in the capillaries along with parasitized erythrocytes.

Other organs

Neither gross nor microscopic pathological changes were noted in any of the other organs. After the 2nd day of infection, parasitized erythrocytes could be found in the blood vessels of the heart, diaphragm, stomach, small and large intestine, tongue, and brain. Terminally, phagocytes could be found in the blood vessels and occasionally wondering about in the connective tissue. The lymphatic tissue of the intestine, consisting of solitary nodules in the lamina propria are seen to undergo the same changes apparent in the peripheral lymph nodes.

DISCUSSION

The pathological response of animals to malarial infection is associated with defense mechanisms involving cellular proliferations and phagocytosis and by degenerative activity evidence by the destruction of cells. Both processes are concurrent and appear to be mutually dependent. Infections with *P. berghei* in the mouse have proved to be excellent for the study of tissue changes and cellular reactions. The course of infection follows neither the exponential curve ascribed to *Plasmodium knowlesi* in *Macacus mulatta*[15] with subsequent death, nor does it resemble the course of *Plasmodium cynomolgi* in *M. mulatta*, which is characterized by a moderately heavy infection followed by spontaneous cure.[6] In the former the curve of infection indicates that only the forces of innate immunity are available to the host to combat the infection and that acquired immunity has no opportunity to take part in defense. In the latter the mechanisms of acquired immunity join those of innate immunity to overcome the infection. In the white mouse, *P. berghei* follows an exponential rise in parasite numbers through the first 4 days of infection, a drop in numbers on the 5th and thereafter a steadily dropping rate of increase until death.[16] This indicates that certain factors of acquired immunity have joined in the defense of the host but, unlike the case of *P. cynomolgi*, are unable to conquer the infection. Therefore, the infection in mice appears to lie between the two previously cited. It permits prolonged stimulation of the elements of defense without the intercession of either drugs or spontaneous cure. This is evidenced by marked hyperplastic changes in the lymph nodes which have not been described heretofore in malarial infections.

The defense reactions of the mouse to *P. berghei* are demonstrated by pigmentation and cellular proliferations, both of which are intimately associated with the destruction of red cells. The degree of response is related to the ability of the host to supply substrate for the parasite, to destroy parasites in red cells, and to replace cells destroyed by

15. Maier, J. and Coggeshall, L. T. 1944, The duration of immunity to *Plasmodium knowlesi* malaria in Rhesus monkeys, J. Exper. Med. **79:** 401–430.
16. Singer, I. 1954, The course of infection with *Plasmodium berghei* in inbred CF 1 mice. J. Infect. Dis. **94:** 237–240.

toxic or mechanical means. Although the mouse shows great potential in regard to all three, it is unable to overcome the infection. As in other malarial infections, the spleen is the organ showing the most immediate and most severe response. Normally, both myelopoiesis and lymphopoiesis occur in the mouse spleen, in contrast to the monkey and man, where extramedullary myelopoiesis can appear but only under conditions of extreme stress. As early as the 1st day after infection, hyperplasia of both the myeloid and lymphoid elements of the spleen is apparent. Through the 6th day, the erythroid tissue becomes far more active than the granulocytopoietic tissue and severe depletion of the white pulp is found. For the remainder of the infection, the spleen is predominantly an erythropoietic organ (plate I, fig. 1 to 7). The lymphoid tissue appears only as bare germinal centers, chiefly containing large lymphocytes and giant large lymphocytes, surrounded by the reticular cells of the transitional zone (plate II, fig. 1). This zone is immediately bordered by deeply basophilic cells of the erythrocyte series. It is highly probable that the young cells of the secondary nodules are actually performing as hemocytoblasts, i.e., are functioning as precursors of erythrocytes.

With the decrease of lymphopoiesis in the spleen, there is a concomitant hyperplasia of the lymphatic tissue in the peripheral and central lymph nodes. The germinal centers of the nodules become extremely active and a generalized depletion of small lymphocytes occurs (plate V, fig. 1 to 4). There is evidence for large amounts of lymphocyte transport and lymphorrhexis. The character of the germinal centers progressively changes. On the 6th day of infection, there are a few mitoses among the medium lymphocytes. Terminally, the centers of the nodules are predominantly large lymphocytes, frequently in mitosis, and large giant lymphocytes. Hence it appears that as the spleen loses its ability to supply lymphocytic cells, the tremendous demand for them stimulates the lymph nodes to greater production. When white pulp hyperplasia occurs in the spleen, it may be ascribed to the immediate presence of the infectious agent. In the lymph nodes, however, parasites are found only in the blood vessels; and the observed hyperplastic changes can only be due to indirect stimulation probably caused by the loss of productive capacity in the spleen of the host. In the chicken and canary where no lymph nodes exist, the lymphatic tissue of the bone marrow becomes hyperplastic;[17] while in the monkey bone marrow, secondary lymphatic nodules are sometimes seen.[5] This has not been observed in the mouse.

Large demands are made on the erythropoietic potential of the host due to the massive destruction of erythrocytes. Not only does the spleen become almost exclusively engaged in the production of erythrocytes, but the bone marrow shows an increase in erythroid elements; and young cells of the erythrocyte series are found in the liver. Megakaryocytes, too, increase in numbers and are found not only in the above mentioned organs but also in the lymph nodes (plate IV). These large cells are probably carried into the liver by the bloodstream from the spleen but in the case of the lymph nodes must have been formed there from existing stem or reticular cells.

Proliferative changes in the liver are confined to the sinusoids and the connective tissue in the periportal areas. They seem to reflect concurrent changes in the spleen. In the early part of the

17. Taliaferro, W. H. 1953, Personal communication.

infection, when hyperplasia and depletion of the lymphatic elements of the spleen are evident, many mononuclear cells are found in the liver (plate VII, fig. 1). As the nature of the spleen becomes more erythroid, the character of the infiltrating cells of the liver changes to those of the erythropoietic series (plate VII, fig. 2).

Parasitized erythrocytes are seen in the blood vessels of all organs from the 1st day of infection. In contrast to the findings of Taliaferro and Cannon[5] in monkeys infected with *Plasmodium brasilianum*, concentration of parasitized erythrocytes occurs in the venous sinuses of the mouse spleen and not in the Billroth cords. A possible explanation for this may lie with differences in the circulatory mechanism of mouse and monkey spleens. By the 6th day of infection, although only 10% of the red cells in the peripheral blood are parasitized, the venules and sinuses of the spleen, liver, and bone marrow are packed with infected erythrocytes (plate III). They appear to be agglutinated and often stuck to the sides of the vessels. This phenomenon is first apparent at the time of, or shortly after, the minor parasitological crisis described elsewhere.[16] It is interesting to observe that most of the parasites retained in these vessels are in the segmenter stage of development, and to note that Eaton[18] had demonstrated the in vitro agglutination of mature intra- and extracellular forms of *P. knowlesi* by immune serum, while immature parasites were unaffected. In the peripheral blood the percentage of segmenters does not rise above 4% of the total number of parasites. This would indicate that certain humoral factors of acquired immunity are operating and that late forms of the schizogonous cycle are retained in the deep tissues.

Although phagocytosis is evident on the 1st day of infection, it does not become extensive until the 6th, when pigmentation changes from a granular nature to heavy clumps. Parasitized erythrocytes, erythrocytes, and cellular debris are avidly ingested. Of the organs studied, the liver shows the largest amount of macrophage activity followed by the spleen, lung, and bone marrow, in that order (plate VI). In the normal liver the sinusoidal endothelium is flat and deeply stained. With the beginning of infection, the endothelial cells swell and a few are seen to contain ingested material. By the 4th day, polyblasts of various sizes are seen in the sinusoids and the transformation of lymphocyte to macrophage can easily be followed. Phagocytosis in the spleen is not as intensive as in the liver and is confined for the most part to the areas immediately surrounding the transitional zone. In other malarial infections[5–7] the spleen is reported to be the most actively phagocytic organ. This is not true in the

18. Eaton, M. D. 1938, The agglutination of *Plasmodium knowlesi* by immune serum. J. Exper. Med. **67**: 857–869.

⟹→

A comparison of the activity in the inguinal lymph node of a normal mouse with that in the inguinal lymph node of a mouse sacrificed on the 16th day of infection.

FIG. 1.—A portion of a lymphatic nodule in the inguinal lymph node of a mouse sacrificed on the 16th day of infection, showing the depletion of small lymphocytes from the marginal zone. ×460.

FIG. 2.—Portion of the germinal center of the same nodule, showing a large amount of cellular hyperplasia and lymphorrhexis. ×1350.

FIG. 3.—A portion of a lymphatic nodule in the inguinal lymph node of a normal mouse, showing the large number of small lymphocytes present. ×460.

FIG. 4.—Portion of the germinal center of the same nodule, showing almost no mitotic activity and little cellular debris or lymphorrhexis. ×1350.

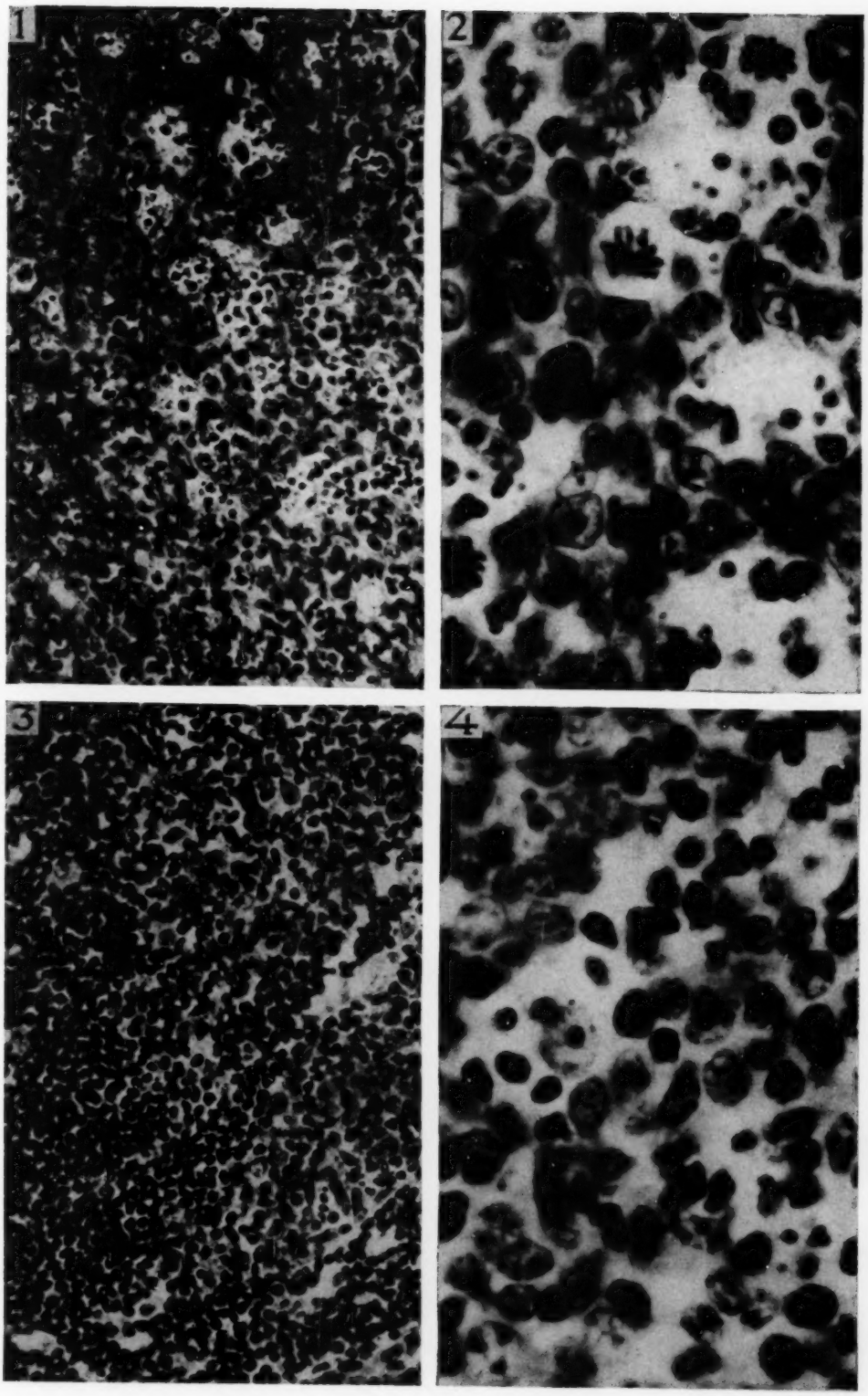


PLATE V

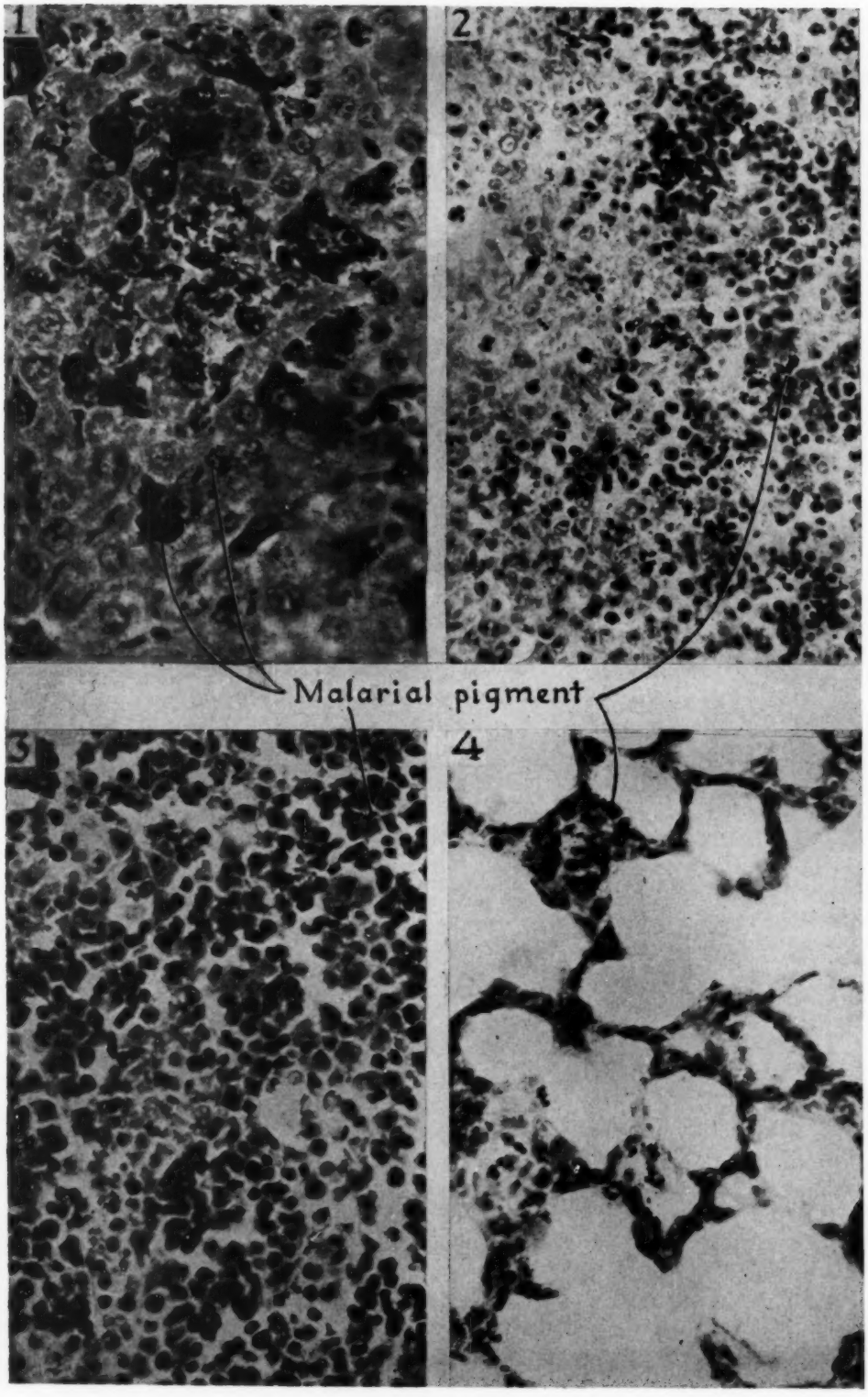


PLATE VI

white mouse and may be related to the loss of lymphopoietic potential in the spleen. After the 6th day of infection, the mouse spleen is able to supply few, if any, lymphocytes. The only cells available for phagocytosis are those mononuclear leukocytes which have access to the spleen parenchyma via the bloodstream, those mononuclears which were supplied by the white pulp earlier in the infection, and those cells of the reticulum which have mesenchymal potencies. This would indicate that the macrophages are neither being replaced nor supplemented as rapidly as if the white pulp continued to show increasing activity.

Phagocytosis in the lung is restricted to cells within the interalveolar capillaries (plate VI, fig. 4). Septal cells are never seen containing ingested pigment. On the 8th day of infection there is a marked enhancement in the number of macrophages in the capillaries and in the amount of phagocytosed pigment and parasites. This increase is probably associated with an opsonic factor of acquired immunity and with greater phagocytopoietic activity of the lymph nodes. A continuous series of cells is seen in the lung capillaries, ranging from lymphocyte or monocyte to polyblasts of various sizes to macrophages.

In the bone marrow a small amount of phagocytosis is evident (plate VI, fig. 3). Up to the terminal part of the infection it is almost exclusively in the sinuses, and only blood histiocytes are responsible. Finally, phagocytes are found in the reticular meshwork; but these, too, are blood histiocytes and appear to have wandered out of the sinuses carrying malarial pigment with them.

In the other organs described earlier, phagocytosis is not marked. Occasionally a macrophage is seen in the tissues but is always of the blood histiocyte type, seemingly associated with a blood vessel that lies close by.

The intensity of phagocytic activity, then, is closely related to the organs of blood filtration and to the macrophages of the blood itself. The liver and spleen contain macrophages which come in contact with the blood; in the lymph nodes and lungs the tissue macrophages are oriented only to remove materials from the lymphatic fluid and the alveoli. This difference in orientation is reflected by intense phagocytosis of malarial pigment in the liver and spleen, while there is an almost complete lack of pigmentation in the histiocytes of the latter organs.

The present work further indicates that phagocytic activity is intimately correlated with changes in the lymphatic tissue and that the lymph nodes are an important source of macrophages. Indirectly, the observation of hyperplastic lymph nodes and concurrent loss of white pulp activity in the spleen mirrors the demand for lymphocytes. In addition, that phagocytic activity in

---

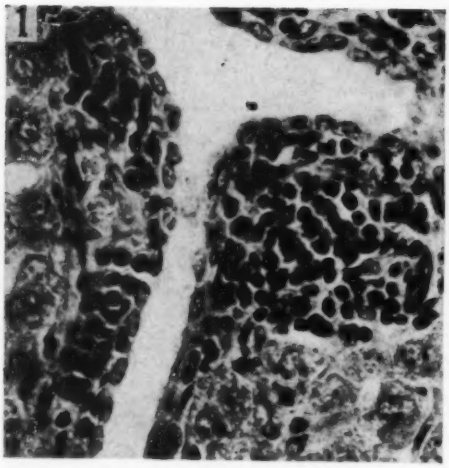
The relative amounts of phagocytosis in the liver, spleen, bone marrow, and lung of a mouse sacrificed on the 16th day of infection. ×480.

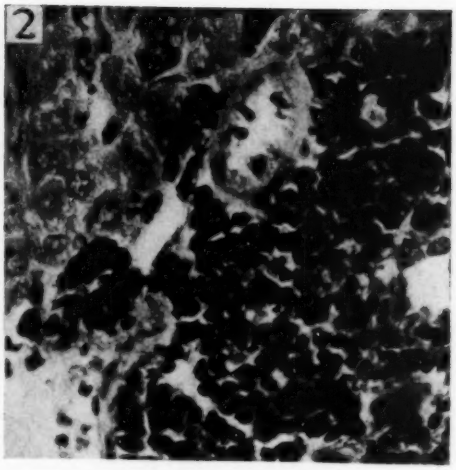
FIG. 1.—The liver, showing heavily pigmented Kupffer cells.

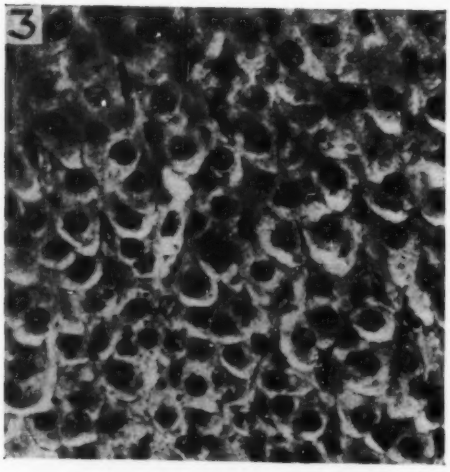
FIG. 2.—The white pulp (left center) and red pulp (right), showing that the heaviest concentration of macrophages with ingested pigment rings the splenic nodules. Note that the macrophages of the spleen contain considerably less pigment than those in the liver.

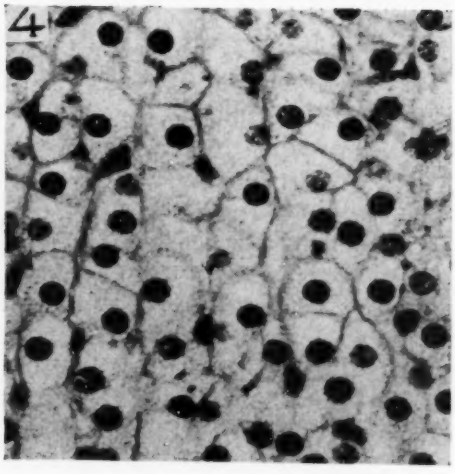
FIG. 3.—The bone marrow, showing relatively little phagocytic activity. Most of the pigmented cells are seen in the sinuses.

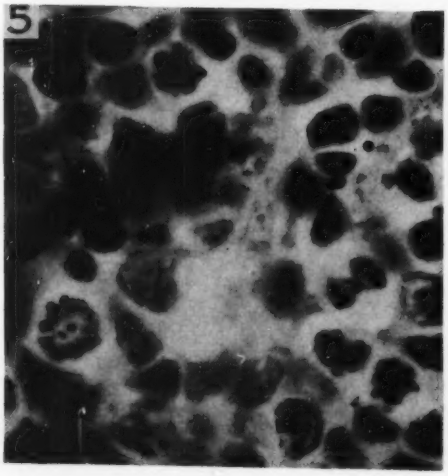
FIG. 4.—The lung, showing greater phagocytic activity than the bone marrow. Note that ingested pigment is found only in cells within the interalveolar capillaries.











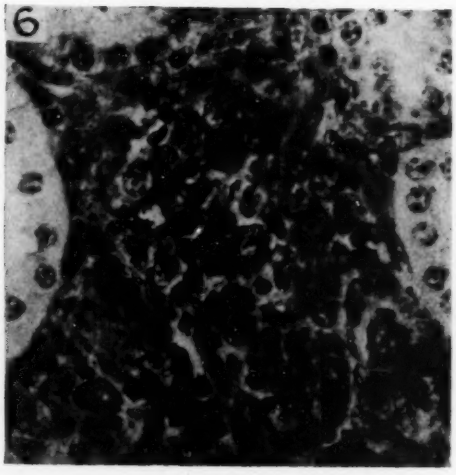


PLATE VII

the spleen is relatively less than that reported in other infections strongly suggests that the loss of lymphopoietic activity in that organ decreases the local supply of macrophages. More directly, there is evidence for the hypertrophy of lymphocytes to polyblasts in the cortical sinuses of the mesenteric lymph nodes (plate II, fig. 2) despite the absence of the organism and its products. This sequence can also be demonstrated in the liver, spleen, bone marrow, and lung. Furthermore, in the reticular organs mitoses in the reticular cells are extremely rare. The great number of macrophages which are mature cannot be due to the mobilization of histiocytes alone, but must be supplemented by the heteroplastic development of small mononuclear cells.

SUMMARY

Observations on the cellular reactions and histopathology in mice with infections of *Plasmodium berghei* have been made. These reactions differ from most other malarial infections in the following ways:

1. The spleen becomes almost wholly erythropoietic, is depleted of lymphatic tissue and loses most of its phagocytic function.

2. With the decrease of white pulp in the spleen, there is a concomitant hyperplasia of lymphatic tissue in the lymph nodes.

3. At the time of, or shortly after, the minor crisis, an increased concentration of parasitized erythrocytes is found in the venous sinuses of the spleen and in the sinuses and venules of the liver and bone marrow, which is highly suggestive of agglutination of these forms by mechanisms of acquired immunity.

4. The liver, and not the spleen, is the most actively phagocytic organ.

The results are discussed in relation to some physiological requirements of the parasite and to certain factors of both innate and acquired immunity.

←

FIG. 1.—Periportal mantling in the liver of a mouse sacrificed on the 6th day of infection. Note the lymphoid character of the infiltrating cells. ×500.

FIG. 2.—Periportal mantling in the liver of a mouse sacrificed on the 13th day of infection. Note the erythroid character of the infiltrating cells. ×500.

FIG. 3.—The fascicular zone of an adrenal of a mouse sacrificed on the 16th day of infection. Note the loss of fatty substance from these cells. The amount of malarial pigment seen here can be compared to that seen in the liver, spleen, bone marrow and lung as shown in plate VI. ×570.

FIG. 4.—The fascicular zone of an adrenal of a normal mouse. ×570.

FIG. 5.—Erythropoiesis in the red pulp of the spleen of a mouse sacrificed on the 16th day of infection. Note the large number of mitotic figures. ×1300.

FIG. 6.—Portion of a kidney of a mouse sacrificed on the 8th day of infection. Note the intertubular accumulation of lymphoid cells. ×550.

A HISTOPATHOLOGIC STUDY OF THE ANTERIOR SMALL INTESTINE OF IMMUNIZED AND NONIMMUNIZED MICE INFECTED WITH *TRICHINELLA SPIRALIS*

JOHN E. LARSH, JR. AND GEORGE J. RACE*

From the Department of Parasitology, School of Public Health, University of North Carolina, Chapel Hill, North Carolina and the Department of Pathology, Duke University School of Medicine, Durham, North Carolina

In a recent study, it was demonstrated in old mice that the immunity to reinfection with *Trichinella spiralis* is general in nature (Larsh, 1953). Because of the evidence obtained in other recent studies in our mice, it was suggested that this immunity results from the action(s) of specific antibodies, probably antiadult antibodies. In addition to the role of humoral factors, there is support for the suggestion that secondary cellular reactions play a role in immunity to certain parasitic worms (Taliaferro, 1940), as was demonstrated clearly in the work of Taliaferro and Sarles (1939) on the immunity of rats to *Nippostrongylus muris*. The intestinal phase of this parasite is somewhat similar to that of *T. spiralis*, hence in attempts to learn more about the immunity of mice to *T. spiralis* it seemed worthwhile to consider the possible role of cellular reactions.

In old mice (5.4 to 6.1 months at necropsy), the mechanisms resulting in the elimination of adult *T. spiralis* at reinfection operate effectively only in the anterior half of the small intestine (Larsh et al, 1952). The rate of elimination of worms from the other areas of the intestine was shown to be similar in immunized and nonimmunized mice. This explains the selection of the anterior small intestine for consideration in the present study. In the likely event that the age of the mouse host is a factor in this localization of the immune response, it was decided to use mice of the same age used in the previous study. The results of the present study have been presented previously in abstract form (Larsh and Race, 1953).

Received for publication September 2, 1953.

* Present address: Peter Bent Brigham Hospital, Department of Pathology, Boston, Massachusetts.

MATERIALS AND METHODS

The various experimental procedures were those described in earlier reports from this laboratory. The mouse strain, the strain of *T. spiralis*, and the methods of isolating larvae for infection and recovering adult worms were those used by Larsh and Kent (1949). The precipitin titers were determined by the procedures employed by Hendricks (1950). Student's *t* test was used to determine the statistical significance of the observed differences in numbers of parasites recovered from the immunized and nonimmunized mice. A probability greater than 0.05 was not

FIG. 1–12.—Small intestine stained with hematoxylin and eosin.

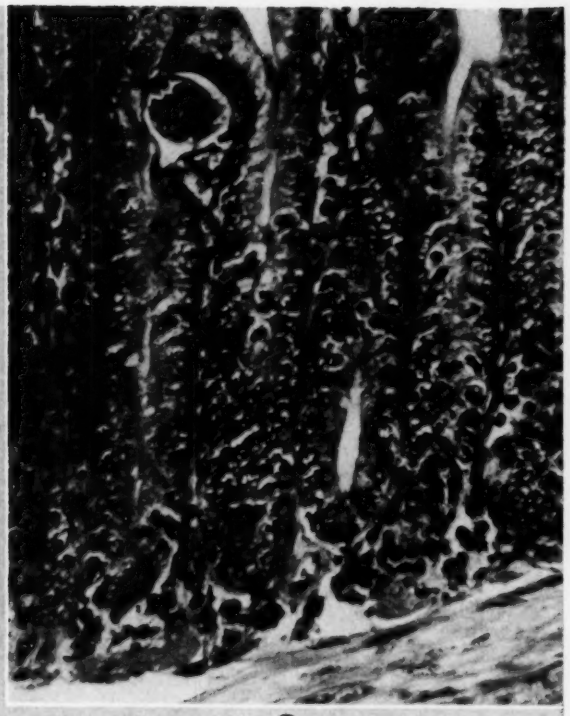
FIG. 1.—Immunized mouse 1 day following challenging infection. Note the worm and very minimal inflammatory reaction. ×348.

FIG. 2.—Nonimmunized control mouse 1 day following infection. Note the worm and relative absence of inflammation. ×348.

FIG. 3.—Immunized mouse 4 days following infection showing an invading adult worm and polymorphonuclear leukocytes throughout the mucosa. ×236.

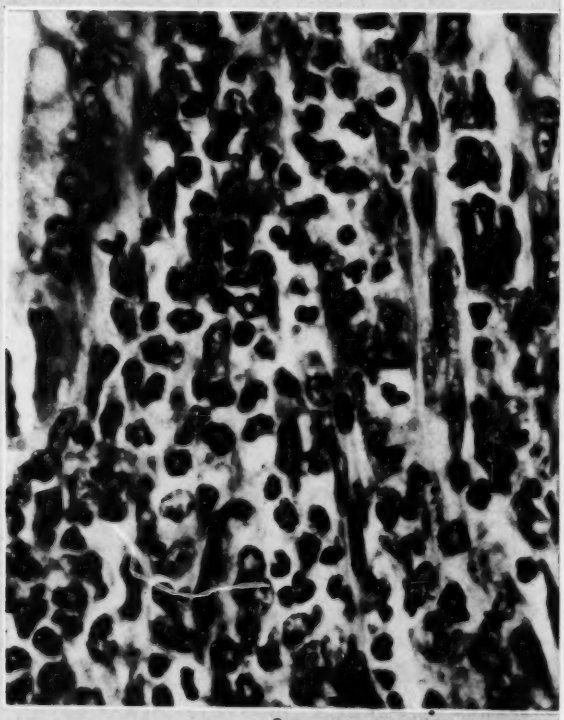
FIG. 4.—Immunized mouse 4 days following infection. The cellular infiltrate is almost entirely polymorphonuclear leukocytic. ×721.

1



2

3



4

considered significant. (All data are on file, Department of Parasitology, University of North Carolina.)

The intestinal tissue for study was prepared as follows. The entire anterior half of the small intestine was removed and fixed unopened in Helly's fluid (formalin, potassium bichromate, and bichloride of mercury). After 15 hours of fixation, the tissue was washed in tap water for 24 hours and then cut into 10 to 15 segments. All segments were embedded in paraffin on end, and histologic cross section preparations 6μ thick were made, which were stained with hematoxylin and eosin.

EXPERIMENTAL PROCEDURES AND RESULTS

Seventy-four mice, eight weeks old, were matched according to sex and approximate weight and divided into experimental and control groups. Of this number, 28 served as experimentals and 46 were used in various control groups.

The experimental mice were given 4 stimulating (immunizing) infections of 200 larvae each at 3-week intervals. To check the viability of the larvae used in these infections, 2 mice, "viability controls," were included at each infection. Thus, a total of 8 mice was used for this purpose. The number of adult worms recovered from these controls 7 days postinfection was within the usual range for the infecting dose of larvae, thereby proving the viability of the larvae administered to the experimental mice. To check on the persistency of the adult worms developing from the larvae given in the stimulating infections, 4 additional mice, "persistency controls," were given doses of larvae in the stimulating infections similar to those given to the experimental mice. These controls received no further infections and were necropsied with the experimental mice (see below). Four weeks after the last stimulating infection, the 28 experimental mice and the remaining 34 previously uninfected controls were given a challenging (test) infection of 400 larvae. At intervals after the challenging infection through the 14th day, certain of the mice in both groups were necropsied and the proximal half of the small intestine was removed for preparation of histologic sections. At 7 days postinfection, 10 experimental mice and 10 controls were bled, to collect serum for the precipitin test, and then were necropsied for counts of the number of adult worms present in the small intestin. The 4 persistency controls were not bled but were necropsied at this time for adult worm counts.

The 10 experimental mice had an average of 140 adult worms, whereas the 10 nonimmunized controls had an average of 340. The 4 persistency controls had very few worms (average, 2), so that for practical purposes it can be assumed that the number recovered from the other mice represented only those developing from the challenging infection given 7 days prior to necropsy. The

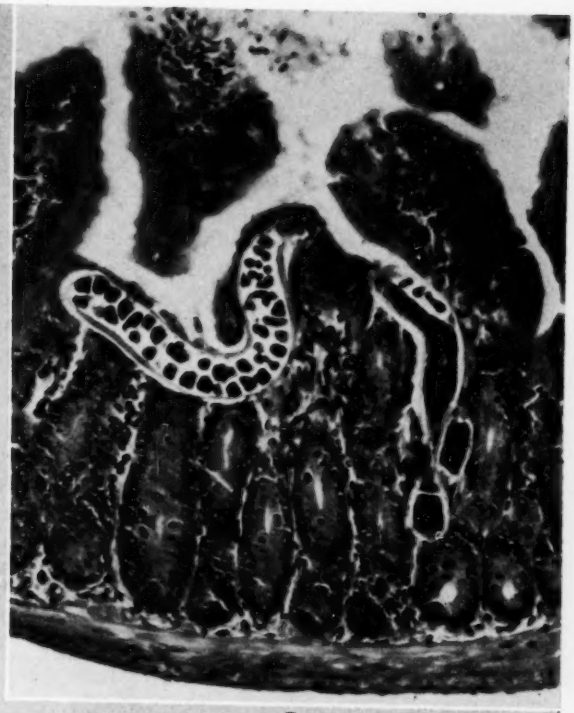
FIG. 5.—Immunized mouse 4 days following challenging infection. Note the mucosal inflammatory reaction and the submucosal infiltration of plasma cells, lymphocytes and polymorphonuclear leukocytes. ×128.

FIG. 6.—Nonimmunized control mouse 4 days after infection. Note the invading adult worm and the minimal inflammatory reaction. ×188.

FIG. 7.—Immunized mouse 6 days following infection. Note the adult worm and the mixed inflammatory infiltrate which consists of polymorphonuclear leukocytes, plasma cells, lymphocytes and a few macrophages. ×179.

FIG. 8.—Nonimmunized control mouse 6 days after infection. The mucosa contains small numbers of polymorphonuclear leukocytes. ×171.

5



6

7

8

probability for the difference in the average number of worms recovered from the experimentals (140) and the controls (340) is 0.01, indicating that acquired immunity had been developed by the experimental mice. In degree, this immunity was quite similar to that reported in the earlier study mentioned above (Larsh et al, 1952). The precipitin titer of the serum pooled from the experimental mice was 1:640, which was that expected from earlier work in our mice (Hendricks, 1952). The titer for the controls, infected for 7 days, was only 1:2, the same titer reported previously for a comparable group of our mice (Baughn, 1952). Thus these results, confirming earlier findings, made it possible to proceed with the tissue study to compare the cellular response in the immunized and nonimmunized mice. Of the 74 mice selected for the entire study, 32 were used, as explained above, to prove that the experimental mice had developed the expected degree of immunity. The remaining 42 mice were used for the tissue study.

The schedule for sacrificing the mice to be used in the tissue study was based on the results of the earlier report (Larsh et al, 1952) indicating the time after reinfection when the worms are eliminated. For the immunized group, 2 mice each were sacrificed 12 and 24 hours, respectively, after reinfection. Thereafter, 2 each were sacrificed daily through the 8th day, making a total of 18 immunized mice used in this phase of the study. For the nonimmunized controls, the same schedule was used but extended to include the 10th, 12th, and 14th days after infection, making a total of 24 controls. As explained above, the study of the cellular changes was limited to the anterior half of the small intestine.

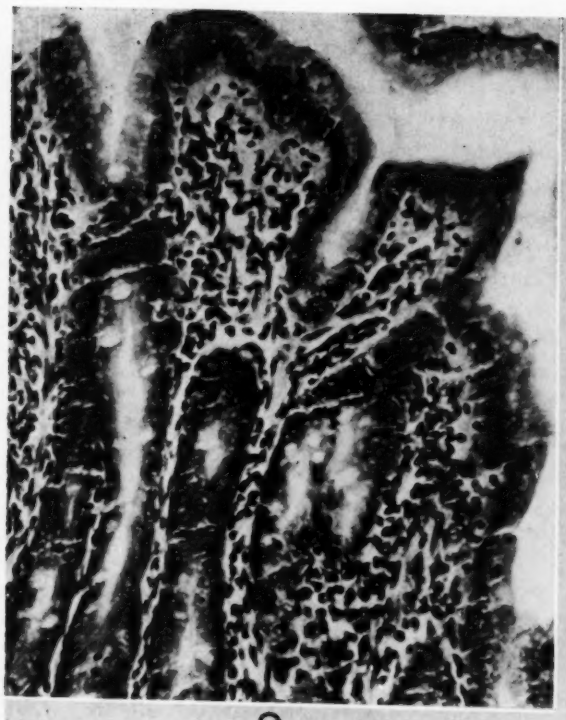
In the immunized mice, three stages of the inflammatory reaction were recognized: (1) a mild, early reaction developing within the first 24 hours, (2) a rapidly developing, severe acute reaction reaching a zenith on the 4th day, and (3) a subsiding subacute or chronic reaction characterized by the presence of mixed mononuclear cells. Within the first 12 to 24 hours, small numbers of polymorphonuclear leukocytes appeared in all layers of the mucosa and around the worms (fig. 1). Subsequently, from the 2nd to the 4th day, the number of polymorphonuclear leukocytes increased greatly in number to reach a maximum at 4 days, when a severe, acute pan mucosal and submucosal inflammatory reaction existed associated with mucosal edema (fig. 3 to 5). Finally, from the 5th to the 8th days the inflammatory reaction diminished in intensity and became mixed mononuclear in type (fig. 7 and 9). As early as 4 days, small numbers of plasma cells and lymphocytes were present, but by the 8th day the inflammatory infiltrate consisted almost entirely of a mixture of

FIG. 9.—Immunized mouse 8 days following infection. The inflammatory reaction is diminished and the predominant cells are mononuclear. ×271.

FIG. 10.—Nonimmunized control mouse 8 days after infection. The mucosa and submucosa are intensely inflamed and are infiltrated with polymorphonuclear leukocytes, plasma cells and lymphocytes. ×279.

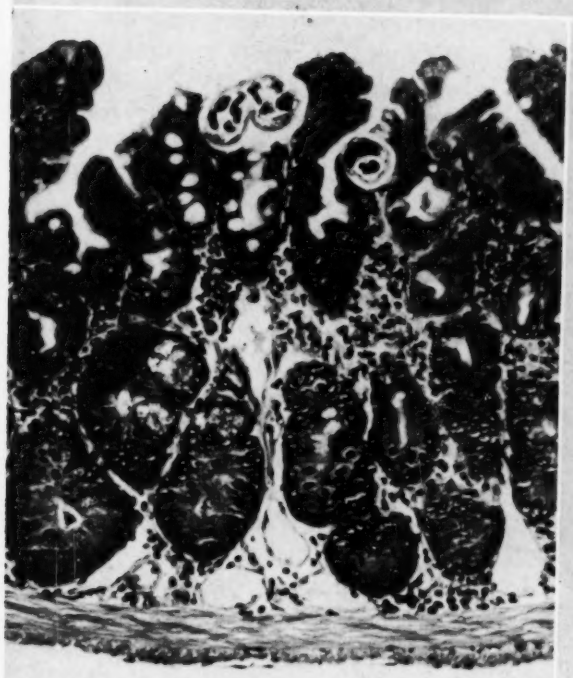
FIG. 11.—Nonimmunized control mouse 10 days after infection. The inflammatory reaction is less intense than in figure 10. The cellular infiltrate is similarly mixed in type. ×205.

FIG. 12.—Nonimmunized control mouse 14 days following infection. Note the adult worm adjacent to the vein. The inflammatory reaction has subsided. The cellular infiltrate consists of plasmacytes, lymphocytes and macrophages with a few polymorphonuclear leukocytes. The lymph node is mildly inflamed. ×162.

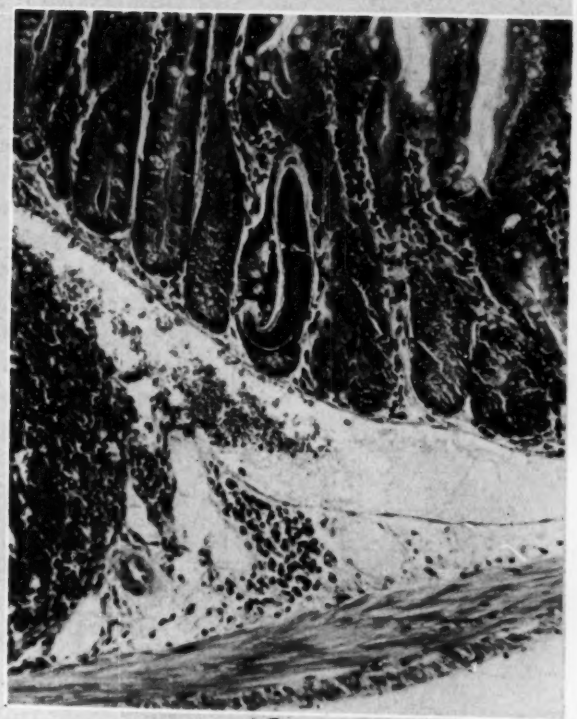


9

10



11



12

plasmacytes, lymphocytes, and macrophages. Eosinophilic leukocytes were seen only rarely in the mucosa and apparently occurred independently of the severity of the inflammation. The worms were located at all depths from the very superficial to the deep mucosa. The worms, most of them with granular material within the cells, did not show the same rate of development as those in the control mice. No protein precipitates were observed around the oral or anal orifices or within the alimentary tract of the worms, but material resembling such precipitate was noted in some of the tissues. The number of polymorphonuclear leukocytes around the worms during the severe acute phase was not increased greatly over the number seen generally in the mucosa.

In the nonimmunized control mice, similarly, three stages in the development of the inflammatory reaction were recognized, although the stages appeared at different times and the reactions were less severe. There was a mild early reaction developing from the 4th to the 6th days, a moderately severe, acute reaction reaching a zenith at approximately the 8th day, and thereafter, lasting through the 14th day, a subacute or chronic diminishing reaction characterized by mixed mononuclear cells. Initially, in the early phase, either no inflammatory reaction was present for the first 4 days, or only a very few polymorphonuclear leukocytes were found immediately adjacent to the worms (fig. 2 and 6). Subsequently, by the 6th day, some polymorphonuclear leukocytes were present, and by the 8th day, the inflammatory reaction reached a zenith when considerable numbers of polymorphonuclear cells were present in the entire mucosa and submucosa (fig. 8 and 10). Finally, by the 10th day, the subacute or chronic diminishing inflammatory reaction was developing, as evidenced by infiltration of moderate numbers of plasmacytes, lymphocytes, and macrophages (fig. 11). On the 14th day the exudate was almost entirely of the mixed mononuclear type (fig. 12). Eosinophils were rare at all times. The worms in nonimmunized mice were larger and showed evidence of maturation not seen in those in the immunized mice. Most of the females contained large numbers of eggs and embryos. No precipitates were observed around or within the worms or in the tissues.

DISCUSSION

The present findings combined with the results of certain of our other studies indicate that acquired immunity to *T. spiralis* in mice is primarily humoral, with secondary cellular reactions or responses associated with the inflammation resulting from reinfection. For an account of the previous evidence that in this host the immunity is generalized, and due probably to the actions of anti-adult antibodies, the reader is referred to a recent report (Larsh, 1953). The present results substantiate the previous conclusions and, in addition, show that certain cellular reactions play a role in this immunity.

In the immunized mice, the appearance of the worms was of particular interest. Within most of them the cells had a granular appearance suggestive of cellular degeneration. All of them, but especially the females, were stunted, as compared with worms of the same age observed in the nonimmunized mice. This agrees with previous observations of other worms in various immunized hosts. The striking degree of interference with the normal development of the adult worms in the present case was anticipated, since smaller worms have been noted in mice after only one small stimulating infection (Rappaport and Wells, 1949). In addition to the smaller

size of the worms, other observations of the present study indicated that in the immune host the normal development of the worms was being hampered. The ovarian and uterine cavities of the females showed fewer eggs and embryos than those in females of the same age in the nonimmunized mice. Moreover, most of the eggs, showing vacuolation and indented edges, appeared to be incapable of normal development. This direct evidence for the interference with normal growth and the apparent interference with the normal reproductive potential of the females, both due presumably to antibody reactions, agrees with previous work on certain other parasitic worms (Taliaferro and Sarles, 1939, and others).

In the intestinal tissue of many of the immunized mice small collections of material were observed. This material had the appearance of protein precipitate as described by Taliaferro and Sarles (1939) in the tissues of rats infected with *N. muris*. In the rat tissues, abundant masses of precipitate were observed, and in many cases this material was demonstrated within the alimentary tract of the worms. There would seem to be no doubt that this precipitate resulted from the antigen-antibody reaction. In the present case, this material was not observed within the worms, as would have been expected from the failure to demonstrate it within worms incubated in antiserum (Oliver-Gonzalez, 1940). As suggested by this worker, this is due probably to the absence of a strongly developed esophageal bulb in *T. spiralis*. This bulb is highly developed in *N. muris*, in which case large amounts of precipitate have been observed in the intestine of worms in vitro as well as in vivo. In any event, the presence in our mouse tissues of material resembling precipitate is further evidence for the role of a humoral factor(s) in the immunity to *T. spiralis*.

In the nonimmunized mice, the worms were much larger than those of the same age in the immunized mice and they appeared to be undergoing normal development. Material resembling protein precipitate was not observed in the intestinal tissue of these mice.

The immunized and nonimmunized mice were inoculated with the same number (400) of larvae in the challenging infection. During the first 4 days after infection, there is, in mice of this age, no significant difference in the numbers of worms present in mice of the two groups (Larsh et al, 1952). Therefore, a difference in worm burden was not a factor in the resulting cellular reactions during this period. The degree of mechanical tissue damage caused by the migrations of the worms appeared to be much greater in the nonimmunized mice. Others have noted this extensive tissue damage following an initial infection (Gursch, 1949). It is likely, therefore, that the much higher antibody titer (1:640) of the immunized mice than that (1:2) of the nonimmunized mice was involved in preventing, partially at least, mechanical damage by the migrating worms. By the direct effects of antibodies, or possibly other humoral factors, the worms in the immunized mice were stunted and partially immobilized (see above), indicating that they would be less capable of injuring the tissue in this way.

Despite the evidence for less mechanical damage produced by the worms in the immunized mice, the inflammation was initiated much sooner after infection and was considerably more severe than that in the nonimmunized mice. Inflammation was evident at the time of the first examination at 12 hours post-infection, suggesting that the reaction was initiated immediately after reinfection. The reaction required only 4 days

to reach its peak level. By contrast, in the controls there was little evidence of inflammation before 4 days postinfection, and at its peak at 8 days the reaction was not as marked as that in the immunized mice at 4 days. It is probable that this more rapid and severe response in the immunized mice was due to the local fixation by antibodies of the irritating antigens (no doubt excretions and secretions of the worms). Evidence for such fixation was the demonstration of precipitate-like material in certain of the tissues. Some feel that the antigen-antibody reaction with resulting fixation probably occurs often without visible precipitates, possibly within certain of the cells (Taliaferro and Sarles, 1939). These workers considered that the antigen-antibody combination elicits more intense inflammation than the antigen alone in a manner similar to, but much milder than, the Arthus phenomenon in the rabbit. There would appear to be strong support in the present case for the suggestion that the antigen-antibody reaction brings about a more intense inflammatory reaction. During the early stages of infection when few, if any, antibodies could have been present, no inflammation was noted in the tissues of the nonimmunized mice despite extensive mechanical damage. On the day prior to the peak level of the response, antibodies in low titer (1:2) were detected in the circulating blood. On the other hand, in the immunized mice with antibodies present at reinfection the inflammatory response apparently was initiated immediately. It seems logical, therefore, to associate in this way the presence of antibodies with the inflammation, and to assume that the fixation of greater quantities of inflammatory irritants would result in a more intense response. It is worth recalling that the height of the inflammatory response in both the immunized and nonimmunized mice was reached just prior to the period when there occurs, in mice of this age, a sudden and significant loss of worms from the same region of the intestine (Larsh et al, 1952). It would seem, therefore, that when the combined direct effects of antibodies and the subsequent indirect effects of certain cellular reactions become great enough, most of the worms are expelled from the anterior half of the small intestine. In mice of the age used here this occurs between 5 to 7 days in the immunized mice and between 11 to 14 days in the nonimmunized mice, the greatest difference in counts occurring at 7 days (Larsh et al, 1952).

As brought out above, there was evidence that the immune phenomena had begun to develop in the nonimmunized mice during the course of the initial infection, as has been shown in other infections (Taliaferro and Sarles, 1939). At about the time antibodies were detectable in the circulating blood of the nonimmunized mice, the inflammation in the anterior half of the small intestine was near its peak. It is known from previous studies in mice of this age that soon thereafter most of the worms are eliminated from this region of the intestine. Therefore, one cannot attribute to age immunity the smaller recovery of adult worms from old mice than from young mice 15 days after initial infection (Riedel, 1950). It is likely that the difference in recovery of worms was due to a more rapid development of acquired immunity in the old mice, as has been reported in another host-parasite combination (Cort and Otto, 1940). In mice, the role of age immunity to *T. spiralis* would, therefore, have to be determined by counts made prior to the sudden loss of worms due to acquired immunity, which occurs between 11 to 14 days post infection (Larsh et al, 1952).

In conclusion, there is now sufficient evidence for the suggestion that in old mice, at least, the immunity to reinfection with *T. spiralis* is a generalized immunity (Larsh, 1953), and that humoral factors, probably antiadult antibodies, are responsible for certain direct, deleterious effects on the worms. There is also evidence that the antigens released by the worms, and the resulting fixation of these antigens by antibodies, elicit a more intense inflammatory reaction than that caused by mechanical damage alone. It seems safe to suggest that the cellular reactions from the resulting inflammation have indirect effects on the worms, i.e., these reactions are secondary to the actions of the humoral factors. Finally, it appears that when these combined effects become great enough, most of the worms are expelled from the proximal half of the small intestine. Although a direct comparison cannot be made with the immunity of rats to *N. muris* as described by Taliaferro and Sarles (1939), especially because of morphological differences of the worms and differences in the life cycles of the two parasites, it is clear that the immune phenomena are quite similar.

Some workers feel that the cellular phase of immunity to *T. spiralis* should be given more consideration (Stoner and Hale, 1952). They noted a sharp reduction in circulating leukocytes in trichinella-immune mice whose immunity was destroyed by $Cobalt^{60}$ gamma whole body irradiation (600 rep). While this observation is not conclusive, because of the many effects of such irradiation, it points up the need for further knowledge of the cellular phase of this immunity.

SUMMARY

The experimental mice given four stimulating infections and controls not infected previously were inoculated with a challenging dose of *Trichinella spiralis* larvae. Seven days later some of the mice of both groups were necropsied. Counts of the numbers of adult worms in the small intestine proved that the experimental mice had developed immunity, and the precipitin titers were those expected from previous work.

The remaining mice in both groups were necropsied at intervals after the challenging infection for histopathologic studies of the anterior half of the small intestine, the only location in which the defensive mechanisms operate effectively in old mice.

The difference in the inflammatory response of the immunized and nonimmunized mice was one of time and severity. Inflammation of a mild degree was evident in the immunized mice at 12 hours postinfection, and it reached a peak at 4 days. By contrast, little or no inflammation was evident in the controls before 4 days, and the peak was not reached until 8 days. The worms in the intestinal tissue of the immunized mice were smaller and less well developed than those of the same age in the controls.

In both groups of mice, the peak of the inflammatory response occurred shortly before a significant loss of worms was noted from the same area of the intestine in comparable groups of an earlier study. Evidence is presented in the *Discussion* for the suggestion that in old mice the immunity to reinfection with *T. spiralis* results from the direct effects of a humoral factor(s) and to indirect effects of cellular reactions associated with the resulting inflammation.

REFERENCES

Baughn, C. O., Jr. 1952, The effect of adrenalectomy on natural and acquired resistance of mice

of *Trichinella spiralis*. J. Elisha Mitch. Sc. Soc. **68**: 207–221.

Cort, W. W. and Otto, G. F. 1940, Immunity in hookworm disease. Rev. Gastroenterology, **7**: 2–13.

Gursch, O. F. 1949, Intestinal phase of *Trichinella spiralis* (Owen, 1835) Railliet, 1895. J. Parasitol. **35**: 19–26.

Hendricks, J. R. 1950, The relationship between precipitin titer and number of *Trichinella spiralis* in the intestinal tract of mice following test infections. J. Immunol. **64**: 173–177.

——. 1952, Studies in mice on the dual antibody basis of acquired resistance to *Trichinella spiralis*. J. Elisha Mitch. Sci. Soc. **68**: 12–26.

Larsh, J. E., Jr. 1953, Studies in old mice to test the hypotheses of local and general immunity to *Trichinella spiralis*. J. Infect. Dis. **93**: 282–293.

Larsh, J. E., Jr. and Kent, D. E. 1949, The effect of alcohol on natural and acquired immunity of mice to infection with *Trichinella spiralis*. J. Parasitol. **35**: 45–53.

Larsh, J. E., Jr. and Race, G. J. 1953, A histopathologic study of the anterior small intestine of immunized and non-immunized mice infected with *Trichinella spiralis*. J. Parasitol. (Aug. suppl.) **38**: 40.

Larsh, J. E., Jr., Gilchrist, H. B. and Greenberg, B. G. 1952, A study of the distribution and longevity of adult *Trichinella spiralis* in immunized and non-immunized mice. J. Elisha Mitch. Sc. Soc. **68**: 1–11.

Oliver-Gonzalez, J. 1940, The in vitro action of immune serum on the larvae and adults of *Trichinella spiralis*. J. Infect. Dis. **67**: 292–300.

Rappaport, I. and Wells, H. S. 1949, Immunity to reinfection in trichinosis. J. Parasitol. (Dec. suppl.) **35**: 14.

Riedel, B. B. 1950, Further studies of the effect of age of mice upon adult *Trichinella spiralis*. J. Parasitol. **36**: 27–28.

Stoner, R. D. and Hale, W. M. 1952, Effect of cobalt^{60} gamma radiation on susceptibility and immunity to trichinosis. Proc. Soc. Exper. Biol. & Med. **80**: 510–512.

Taliaferro, W. H. 1940, The mechanism of acquired immunity in infections with parasitic worms. Physiol. Rev. **20**: 469–492.

Taliaferro, W. H. and Sarles, M. P. 1939, The cellular reactions in the skin, lungs and intestine of normal and immune rats after infection with *Nippostrongylus muris*. J. Infect. Dis. **64**: 157–192.

GROWTH OF NEUROTROPIC VIRUSES IN EXTRANEURAL TISSUES

V. SURVEY OF TISSUES OF VARIOUS ANIMAL SPECIES FOR CAPACITY TO SUPPORT MULTIPLICATION OF POLIOMYELITIS VIRUS IN VITRO

C. A. EVANS, VELMA C. CHAMBERS, WAYNE M. SMITH, AND PAMELA H. BYATT*

From the Department of Microbiology, University of Washington School of Medicine, Seattle, Washington

Since the successful cultivation of poliomyelitis virus in vitro by Enders and his associates, the use of tissue cultures in the study of this virus has become a well established and fruitful practice. One of the tissues most commonly used at the present time is monkey testicular tissue.[1–12] The virus grows well in human embryonic skin and muscle.[13–16] Numerous studies have also demonstrated multiplication of the virus in human foreskin,[14] kidney,[17] uterus,[18] and embryonic intestine[13,16] and possibly placenta.[16] In the experiments reported in this paper, various kinds of tissues from laboratory and domestic animals were tested to determine their relative value for the propagation of poliomyelitis virus in suspended cell cultures.

Received for publication September 2, 1953.

Aided by a grant from the National Foundation for Infantile Paralysis.

* Present address: Department of Infectious Diseases, School of Medicine, University of California, Los Angeles.

METHODS AND MATERIALS

Enders[19] has published a review of early papers on the subject of cultivation of poliomyelitis viruses in tissue culture. Details of the procedures employed in the cultivation of these viruses in various human and simian tissues in suspended cell culture and roller tube culture are described by several authors.[1,6,8,18–21]

Flask cultures.—Approximately 0.04 g. of a heavy suspension of finely minced tissue was

1. Smith, W. M., Chambers, V. C. and Evans, C. A. 1951, Proc. Soc. Exper. Biol. & Med. **76:** 696–700.
2. Syverton, J. T., Scherer, W. F. and Butorac, G. 1951, Proc. Soc. Exper. Biol. & Med. **77:** 23–28.
3. Ledinko, N., Riordan, J. T. and Melnick, J. L. 1951, Proc. Soc. Exper. Biol. & Med. **78:** 83–88.
4. Youngner, J. S., Ward, E. N. and Salk, J. E. 1952, Am. J. Hyg. **55:** 291–300.
5. Youngner, J. S., Ward, E. N. and Salk, J. E. 1952, Am. J. Hyg. **55:** 301–322.
6. Ledinko, N., Riordan, J. T. and Melnick, J. L. 1952, Am. J. Hyg. **55:** 323–338.
7. Riordan, J. T., Ledinko. N. and Melnick, J. L. 1952, Am. J. Hyg. **55:** 339–346.
8. Youngner, J. S., Lewis, L. J., Ward, E. N. and Salk, J. E. 1952, Am. J. Hyg. **55:** 347–356.
9. Evans, C. A., Smith W. M. and Chambers, V. C. 1951, Bact. Proc. p. 93.
10. Syverton, J. T. and Scherer, W. F. 1952, J. Exper. Med. **96:** 355–367.
11. Scherer, W. F. and Syverton, J. T. 1952, J. Exper. Med. **96:** 369–388.
12. Scherer, W. F. and Syverton, J. T. 1952, J. Exper. Med. **96:** 389–400.
13. Enders, J. F., Weller, T. H. and Robbins, F. C. 1949, Science **109:** 85–87.
14. Weller, T. H., Robbins, F. C. and Enders, J. F. 1949, Proc. Soc. Exper. Biol. & Med. **72:** 153–155.
15. Robbins, F. C., Enders, J. F. and Weller, T. H. 1950, Proc. Soc. Exper. Biol. & Med. **75:** 370–374.
16. Milzer, A., Levinson, S. O., Vanderboom, K. and Adelman, P. 1950, Proc. Soc. Exper. Biol. and Med. **74:** 136–145.
17. Robbins, F. C., Enders, J. F., Weller, T. H., and Florentino, G. L. 1951, Am. J. Hyg. **54:** 286–293.
18. Robbins, F. C., Weller, T. H. and Enders, J. F. 1952, J. Immunol. **69:** 673–694.
19. Enders, J. F. 1952, J. Immunol. **69:** 639–643.
20. Smith, W. M., Chambers, V. C. and Evans, C. A., 1950, Northwest, Med. **49:** 368–373.
21. Weller, T. H., Enders, J. F., Robbins, F.C. and Stoddard, M. B., 1952, J. Immunol. **69:** 645–671.

deposited on a cellophane disc in the bottom of a 50-ml Erlenmeyer flask. A virus inoculum of 0.04 or 0.4 ml was added to the tissue and allowed to stand for 5 minutes. Fluid medium was then added to make a total fluid volume of 4 ml. This brought the ratio of tissue to fluid to approximately 1:100. The composition of the medium employed is given in a later section of this paper. A mixture of 5% CO_2 and 95% air was used to flush the gaseous phase, and the flask was closed with a rubber stopper. The cultures were incubated at 35 to 37 C and the fluid was changed every 3rd or 4th day by removing about 3.6 ml of fluid and replacing it with an equal volume of freshly prepared fluid medium. Cultures were gassed each time they were opened. The virus was subcultured by transferring 0.04 or 0.4 ml of fluid from previous cultures into flasks containing freshly prepared tissue. The harvested fluids were either injected immediately into susceptible animals to test for the presence of virus or glass-sealed in pyrex glass vials and stored in the dry ice chest for injection at a later date.

Roller tube cultures.—Roller tube cultures were used in this study principally to test the viability of tissues used in flask cultures. They were prepared by incorporating tissue explants from flasks in a thin layer of coagulated plasma on the inner wall of the tube. The cultures were observed for outgrowth of fibroblasts from the explants during a period of 2 weeks.

Roller tube cultures were also used in studies of three cell strains obtained from Dr. G. O. Gey and Dr. W. R. Earle. These cell lines were of rat, mouse and human origin, and had been propagated in vitro for many years. They were tested for their capacity to support the growth of poliomyelitis virus. These cells were usually cultivated directly on the inner glass surface of roller tubes without the use of plasma. Cultures were incubated at 35 to 37 C until cellular proliferation had become well established. They were then inoculated with 0.05 or 0.1 ml of virus suspension. Incubation was continued for a period of 2 weeks, during which cultures were observed for degeneration of cells. Tissue culture fluids harvested during this period were tested for presence of virus by animal inoculation.

Tissue culture medium.—The following ingredients of the tissue culture medium used were used in various combinations: ox serum ultrafiltrate (SUF), obtained from Microbiological Associates, Inc., horse serum (HS), human umbilical cord serum, chick embryo extract ($ChEm_{50}$), beef embryo extract (BEM_{50}), Hanks' balanced salt solution (BSS),[22*] and chicken plasma. Plasma was prepared from heparinized blood from mature roosters starved 48 hours before bleeding. Lyophilized commercial chicken plasma (Difco) was also employed with entirely satisfactory results. The chick embryo extract consisted of finely minced 9- to 10-day-old embryos diluted with an equal volume of Hanks' BSS, centrifuged and frozen. The horse and human serums were usually heated at 56 C for 30 minutes and stored at 4 C. One hundred units of penicillin and 100 μg of streptomycin per ml were added to both chick embryo extract and serums before storage.

The medium routinely employed for suspended cell cultures consisted of 25% SUF and 75% Hanks' BSS. Salk's "Medium C,"[8] consisting of 10% $ChEm_{50}$, 10% inactivated horse serum, 20% SUF and 60% Hanks' BSS, was usually employed for roller tube cultures of testicular tissue. Earle's medium,[23] used for L strain cells, was composed of 20% $ChEm_{50}$, 40% inactivated horse serum and 40% saline. (Hanks' solution was used rather than Earle's.) Gey's medium[24] or modifications of it were employed for all other stock fibroblast cultures, including Gey's T-333 rat strain and D_1Re human sarcoma strain and our human embryonic strains. This medium consisted of 10% of either BEm_{50}, or $ChEm_{50}$, 50% of either horse serum or human umbilical cord serum, and 40% Hanks' BSS. Penicillin, 100 units per ml. and streptomycin, 100 μg per ml, were added to all mediums. A mixture of 5% CO_2 and 95% air was added as the gaseous phase of flask cultures but was not generally employed in the case of roller tube cultures.

Tissue.—Human tissue was obtained from surgical operations and fetal tissue from abortions. Tissues from other species were obtained by surgical excision from the living animal or from animals soon after death.

Promptly after excision, tissues were ordinarily cut into pieces several millimeters in diameter and immersed in Hanks' BSS or tissue culture

---

* Formula for Hanks' BSS obtained from Hanks' publication[22] differs slightly from that obtained from a pamphlet distributed by Microbiological Associates, Inc. The discrepancy involves the amount of $MgSO_4 \cdot 7H_2O$,—2 g per l in the former and 1 g per l in the latter. Both solutions have been used by us without any noticeable difference in results.

22. Hanks, J. H. and Wallace, R. E. 1949, Proc. Soc. Exper. Biol. & Med. **71**: 196–200.
23. Earle, W. R. 1943, J. Nat. Cancer Inst. **4**: 165–212.
24. Gey, G. O. and Bang, F. B. 1951, Tr. New York Acad. Sc. Ser. II, **14**: 15–24.

medium. Tissue to be used in flask cultures was minced with small stainless steel scissors or by chopping with Bard-Parker scalpels. During mincing the tissue fragments were washed several times with Hanks' BSS or with tissue culture medium. Minced tissue fragments were approximately 1 mm in diameter. Explants for roller tube cultures were usually dissected individually from the larger tissue mass and were 0.5 to 1 mm in diameter. Processing of tissue was usually completed within 2 hours after tissue was obtained. All solutions used for storing, transporting and processing tissues contained 100 units of penicillin and 100 μg of streptomycin per ml.

Viruses.—In testing for the capacity of virus to multiply in cultures of various tissues, the initial inoculum was in all instances a suspension of infected central nervous system (CNS) in Hanks' BSS or tissue culture medium. Large pools of each virus were dispensed in small vials, preserved by freezing and used over a period of many months. Virulence for mice and for monkeys was tested periodically.

The Hof. strain of poliomyelitis virus was received from Dr. A. B. Sabin in May, 1949. This virus was originally isolated from stools and throat swabs of a patient in Cincinnati in 1947, and was found to be type 1.[25] Material received from Dr. Sabin represented the third passage in rhesus monkeys. The virus used for tissue culture experiments represented the second monkey passage in this laboratory.

Mahoney virus, also a type 1 poliomyelitis virus,[25] was chosen for certain experiments because it is highly destructive to fibroblasts in tissue culture. It was received from Dr. Jonas E. Salk, August, 1951, as a 20% suspension of infected monkey spinal cord. Material from the first monkey passage in this laboratory was employed in the tissue culture experiments.

Lansing poliomyelitis virus (type 2)[25] was originally isolated and described by Armstrong.[26] The Lansing virus used in the present experiments was obtained from Dr. J. F. Enders in December, 1948, and has been maintained since that time by numerous passages in the central nervous system of mice and one passage in the spinal cord of a cynomolgus monkey. Suspensions of mouse CNS were used to infect tissue cultures.

The Y-SK strain of type 2 poliomyelitis virus is more destructive to fibroblasts in testicular tissue culture than is Lansing virus.[3,5]

Our Y-SK virus was received from Dr. A. B. Sabin in December, 1950, as a suspension of infected mouse CNS. This virus was passed several times in mice and monkeys during the period of the present studies.

In order to estimate the extent of multiplication of virus in tissue cultures, each change of fluid in an infected culture and each subculture to new tissue were estimated to cause a tenfold dilution of original virus inoculum. In periodic titrations of fluid from cultures of tissue in which the virus did not multiply, it was found that the virus decreased and disappeared as expected on the basis of the calculated dilution.

EXPERIMENTAL RESULTS

Cynomolgus testicular tissue in flask cultures

Minimal infective dose of virus for tissue cultures.—In 3 experiments a 10^{-4} dilution of Hof. virus was infective for cynomolgus testicular tissue in flask cultures; a 10^{-5} dilution in 1 experiment and a 10^{-6} dilution in 2 experiments failed to infect testicular tissue. In vivo experiments showed Hof. virus to be infective for 12 out of 15 monkeys inoculated intracranially with a 10^{-4} dilution and 1 out of 7 monkeys inoculated with a 10^{-5} dilution. In 13 experiments with Y-SK virus, the minimal infective dose of virus for tissue cultures appeared to be in the range of 1 to 10 ID_{50} for mice injected intracerebrally.

Amount of virus produced.—The amount of Hof. virus produced in tissue culture was approximately the same, per milligram of tissue, as in infected monkey spinal cord. This means that in a flask containing 4 ml of fluid and approximately 40 mg of infected tissue the fluid of the culture was infective for monkeys when further diluted to 10^{-2} or 10^{-3}. This was equal to a 10^{-4} or 10^{-5} dilution of the infected tissue. It is significant that the virus used as inoculum had not previously been grown in tissue cultures.

Comparison of virus multiplication in

25. The Committee on Typing of the National Foundation for Infantile Paralysis 1951, Am. J. Hyg. **54**: 268–274.
26. Armstrong, C. 1939, Pub. Health Rep. **54**: 1719–1721.

mature and immature cynomolgus testicular tissue in suspended cell cultures.—Mature testicular tissue (spermatogenesis present) was used in 8 experiments and immature tissue in 14 experiments. No difference was seen between the minimal infective doses required to infect the 2 types of tissue. The amount of virus produced in cultures of mature tissue was approximately equal to that produced in immature tissue. It is therefore clear that spermatogenesis is not required for multiplication of poliomyelitis virus.

Further evidence that germinal cells are relatively unimportant for the growth of virus was obtained by the use of tissues from a monkey exposed to 300 r of X-ray irradiation several months previously. The Hof. strain of poliomyelitis virus was maintained through 3 passages in such tissue from testes of 2 irradiated monkeys. The amount of virus produced was equal to that produced in testicular tisssue from normal animals. Histological study of 3 testes employed showed little remaining germinal tissue. Lansing virus multiplied less abundantly but was maintained through 4 passages, involving a calculated dilution of 10^{-10}, in irradiated cynomolgus testicular tissue.

Comparison of different mediums in flask cultures.—The following mediums were employed in flask cultures: (1) bovine SUF, 1 part; Hanks' BSS, 3 parts; this medium was used in most experiments; (2) medium "1" plus 1% chicken plasma; (3) Hanks' BSS; (4) Hanks' BSS, 9 parts, chick embryo extract, 1 part; (5) Hanks' BSS, 95%, horse serum, 5%.

Poliomyelitis virus grew in cynomolgus testicular tissue with all five mediums. There was no striking difference in the amount of virus produced. The tests were designed to reveal only marked stimulation or inhibition of virus multiplication and should not be interpreted as indicating that these mediums are all equally good. It should be pointed out that a significant amount of nutrient substance was undoubtedly provided to surviving cells by the degeneration of cells inside the testicular tubules.

Cytonecrosis and pH changes not useful indications of infection in flask cultures of testicular tissue.—In spite of many attempts, no method was found to determine whether a flask culture of testicular tissue was infected except by subinoculation of tissue culture fluid into animals or into roller tube cultures. The pH changes used by Robbins and his associates in work with human embryonic tissue[15] were not found to be a reliable indication of infection, presumably because a substantial portion of the testicular tissue remained living and metabolically active in the flasks of infected cultures. The medium employed by Robbins probably was not as highly buffered as ours and therefore was more suitable for using pH changes as a measure of tissue viability.

Wide fluctuations in pH occurred in the fluid phase of our suspended cell cultures, depending upon the amount of tissue and the metabolic state of the tissue. A pH of 7.8 to 8.0 was usually observed during the 1st day or 2 of incubation of roller tube cultures with mediums containing serum. The addition of 5% carbon dioxide and 95% air mixture to the gaseous phase of flask cultures resulted in a pH of about 7.4 for the 1st day or 2 of incubation. Actively metabolizing tissue caused a fall in pH to a level of 6.8 or lower after incubation for 3 or 4 days. The decline in pH was presumably due to the accumulation of acids resulting from the utilization of glucose by the tissue. Large amounts of tissue caused a more rapid fall in pH than did small amounts of

tissue. When tissue died, a rise in pH to 7.8 or 8.0 commonly occurred, presumably as a result of autolytic changes.

In order to ascertain whether tissue remained viable in flask cultures, fragments were periodically transferred to roller tube cultures or to slide cultures, using the Maximow double-coverslip method. The evidence used for judging viability was the development of a zone of fibroblasts. Table 1 gives the results of some of these tests. When testicular tissue fragments were tested after a week or two of incubation with Hof. virus in a flask culture, from 50 to 100% of explants were found to be viable.

Rhesus testicular tissue

Rhesus testicular tissue was much less satisfactory than cynomolgus tissue for propagation of poliomyelitis viruses in suspended cell cultures. In 7 experiments, 2 with Hof., 3 with Lansing and 2 with Y-SK, virus failed to multiply in 4. In 2 of the remaining 3 experiments the concentration of virus was relatively low. In 1 experiment, Hof. virus multiplied exceptionally well. It was carried through 3 passages in testicular tissue derived from adult rhesus monkeys that had been given 300 r of X-ray irradiation 4 to 8 months earlier to produce atrophy of germinal epithelium. Histological studies were made on 2 of the 3 testes employed and showed almost complete absence of germinal cells. Tubules were lined with Sertoli cells. There was no inflammation. Tissue culture fluid harvested on the 15th day of the 3rd tissue culture passage infected all of 3 test monkeys in a dilution of 10^{-2}. This represented a dilution of 10^{-12} of the original inoculum.

Testicular tissue of animal species other than man and monkeys

Following the successful cultivation of poliomyelitis virus in flask cultures of

TABLE 1.—*Viability of monkey tissues after incubation in flask cultures with or without poliomyelitis virus.*

Tissue	Virus inoculated into flask	Flask medium*	Tissue viability Viable explants/total explants		Multiplication of virus‡
			6 to 9 days†	10 to 21 days†	
Cynomolgus testes, mature	No virus	1	17/17	27/34	
	Hof. 10^{-1}	1	13/13	8/29	Not tested
	No virus	1	11/11	9/14	
	Hof. 10^{-2}	3	3/6	11/11	Multiplication
Cynomolgus testes, immature	No virus	1		7/16	
	Hof. (heat killed)	1		15/15	
	Hof. 10^{-2}	1		14/23	Multiplication
	Y-SK 10^{-1} tissue culture virus	1	0/5, 0/8, 7/11		Survival
	Y-SK 10^{-1} tissue culture virus	4	12/13		Survival
	Y-SK 10^{-1} tissue culture virus	5	12/12		Survival
Rhesus testes, immature	No virus	1	0/17	0/14	
	Hof. (heat killed)	1	4/7	0/23	
	Hof. 10^{-2}	1	0/12	0/13	No multiplication
Cynomolgus ileum	No virus	1, 2, 3	3/4, 3/8, 6/7	0/9, 4/10, 6/11	
	Hof. 10^{-2}	2	2/4	0/3	Multiplication through T_3‖
Cynomolgus bladder	No virus	1, 2, 3	0/10, 2/9, 1/7	0/14, 0/10, 1/8	
	Hof. 10^{-2}	2	0/9	0/13	Multiplication through T_3 Virus disappeared in T_3
Cynomolgus heart	No virus	1, 2, 3	0/12, 0/15, 0/10	0/47, 0/39, 0/18	
	Hof. 10^{-2}	2	0/17	0/46	Virus disappeared in T_2

Explants were transferred to roller tubes or slide cultures and observed for fibroblastic outgrowth as evidence of viability.

* Medium "1" is regular flask medium (1 part of beef serum ultrafiltrate and 3 parts of Hanks' balanced salt solution); medium "2" is regular flask medium plus 4% beef embryo extract in equal parts Hanks' (BEm_{50}); medium "3" is regular flask medium plus 1% chicken plasma; medium "4" is Hanks' balanced salt solution; medium "5" is 10% chick embryo extract ($ChEm_{50}$) and 90% Hanks' solution.

† Days of incubation in flasks prior to transfer to roller tube or slide culture. Inoculation of virus into flask cultures was accomplished on the first day. Each fraction represents one experiment.

‡ "Survival" indicates that virus persisted in tissue cultures, but the experiment did not involve a sufficient number of subcultures and changes of medium to demonstrate whether multiplication occurred.

‖ T_3 and T_2 refer to the second and third passages of virus in tissue cultures.

cynomolgus testicular tissue, a series of experiments was carried out to test for multiplication of this virus in similar cultures of testicular tissue from other animal species. The Lansing and the Hof. strains were chosen for this work. In view of our experience with rhesus testicular tissue, it was recognized that to determine whether tissue from a given animal species would support the growth of poliomyelitis virus, it would be necessary to carry out multiple tests, preferably with each of the 2 virus strains. At the time these experiments were performed, it was necessary to use time-consuming animal inoculation tests to determine the virus content of tissue culture fluids, since suitable methods for detecting virus directly in tissue cultures had not been developed.

Poliomyelitis virus of the Lansing and Hof. strains did not multiply in testicular tissue from mice, hamsters, guinea pigs, rats, rabbits, dogs and bulls. Table 2 contains a summary of the experiments conducted on such tissues. It should be emphasized that in view of our irregular results in experiments with rhesus testicular tissue in suspended cell cultures, the negative results with tissue from other animal species do not constitute conclusive evidence that these tissues are not susceptible. These data indicate only that since the virus did not multiply in our experiments, the tissues concerned are substantially less suitable than cynomolgus testicular tissue for cultivation of poliomyelitis virus in suspended cell tissue cultures.

In evaluating the negative results obtained in this portion of the work, particular attention should be paid to the tests for "viability" of explants removed from flask cultures. This was

TABLE 2.—*In vitro tests for multiplication of poliomyelitis virus in flask cultures of testicular tissue of mice, hamsters, guinea pigs, rats, rabbits, dogs, and bulls.*

Source of testicular tissue*	Number of tests	Virus inoculated into flasks		Tissue viability Viable explants/total explants		Multiplication of virus
		Lansing	Hof.	6 to 9 days†	10 to 14 days†	
Mouse	5	10^{-1}				Negative
Mouse, irradiated testes	3	10^{-1}, 10^{-2}		1/12, 3/10, 9/18		Negative
Mouse, irradiated testes	1	No virus		4/4		
Mouse, irradiated testes, immature	3	10^{-1}, 10^{-2}		—, 4/4, 7/7		1 negative 2 questionable
Mouse	1		10^{-1}			Negative
Hamster	4	10^{-1}				Negative
Hamster	1		10^{-1}			Negative
Hamster, cortisone	2	10^{-1}		30/30, 8/14	0/18, 0/10	Negative
Hamster, cortisone	2		10^{-1}	23/23, 5/10	—, 9/40	Negative
Hamster, cortisone	2	No virus		6/14, 0/20	0/10, —	
Guinea pig	2	10^{-1}				Negative
Rat	1	10^{-2}		2/16	0/13	Questionable
Rat	1		10^{-2}	0/12	0/11	Negative
Rat	1	No virus		0/8	0/9	
Rabbit	1	10^{-2}		4/14	4/19	Negative
Rabbit	1		10^{-2}	3/7	0/12	Negative
Rabbit	1	No virus		3/7	1/7	
Dog	3	10^{-1}, 10^{-2}		12/13, 14/14, 15/16		2 negative 1 questionable
Dog	3		10^{-1}, 10^{-2}	14/14, 17/17, 13/14		Negative
Dog	2	No virus		15/16, 17/20		
Bull	2	10^{-1}				Questionable
Bull	1	10^{-2}				Negative
Bull	1		10^{-1}			Negative

* All testicular tissue was from mature animals except the mouse tissue which is indicated as "immature."

† Days refer to the number of days tissue had incubated in flask culture before fragments were removed to slide or roller tube culture. Each fraction represents one test.

Each "test" represents a separate attempt to propagate virus in tissue culture, starting with an inoculum of infected CNS tissue.

Virus inoculum was 0.4 ml of infected CNS suspension. This was diluted to 4 ml by the tissue culture medium. Medium was renewed at intervals of 3 or 4 days, producing further 10-fold dilutions.

Lansing inoculum was titrated in mice at the time tissue culture experiments were started. The ID_{50} calculated from combined results of titrations was $10^{-5.1}$.

The ID_{50} of Hof. inoculum for monkeys was approximately $10^{-4.4}$.

Tests for viability were made by transferring explants from flask cultures to roller tube or slide cultures. If fibroblastic outgrowth resulted an explant was recorded as viable. Presumably some explants contained surviving cells but did not put out fibroblasts.

actually not a test for survival, but for capacity of tissue to produce an outgrowth of fibroblasts. If fibroblastic outgrowth occurred from a majority of the explants, the experiment has substantially more significance, in our opinion, than if this did not occur. In the case of such tissues as rat testes, which showed little "viability," it would seem particularly important that future attempts at poliomyelitis virus cultivation should involve the use of a more enriched medium, and one in which preliminary tests showed that fibroblastic outgrowth would occur.

The results of some of the Lansing experiments are listed in table 2 as questionable because scattered deaths of the mice occurred in tests for virus in tissue culture fluids. It was not determined whether virus was multiplying at a level below that readily detectable by animal inoculation, or whether the deaths of mice were all due to other causes. The results indicate that the virus either did not multiply or multiplied at a very low level. Some of the published experiments by others on cultivation of poliomyelitis viruses in tissue from human foreskin have shown that the virus may be maintained for several passages at a level not readily detectable by animal inoculation.[21]

It has been reported that cortisone increases the susceptibility of hamsters to poliomyelitis virus of type II (MEF_1).[27] In 2 experiments, testicular tissue from hamsters previously treated with cortisone failed to support the multiplication of poliomyelitis virus. In 1 experiment the hamster received 5 mg of cortisone 4 hours before orchiectomy. Cultures of this testicular tissue were inoculated with Lansing and Hof. strains of virus. In the second experiment 4 injections of cortisone, totaling 16 mg, were administered over a period of 45 hours prior to removal of the testes. Lansing virus was used as inoculum for cultures of testicular tissue from this animal. No multiplication of virus occurred in either experiment.

Human and simian tissues other than testicular tissue

During the course of these studies increasing significance was given to tests for survival of tissues in flask cultures, as indicated by ability of explants to produce a zone of fibroblastic outgrowth when transferred from flask cultures to more enriched mediums. Representative data concerning survival of some of the tissues of monkey origin are presented in table 1.

Cynomolgus skeletal muscle.—In 6 experiments, 3 with Hof. and 3 with Lansing virus, no multiplication occurred in cynomolgus skeletal muscle in flask cultures. Tissue from three monkeys was employed.

Cynomolgus cardiac muscle.—Hof. virus appeared to multiply in the primary tissue culture in one experiment with cardiac muscle and survived through the primary tissue culture of a second experiment, but disappeared in the first subculture of both experiments.

Cynomolgus urinary bladder.—In tissue cultures of urinary bladder, Hof. virus underwent limited multiplication in two experiments. It disappeared in the first subculture of the first experiment and in the second subculture of the second experiment. It appears that susceptibility of this tissue is of a lower order and more irregular than that of testicular tissue of cynomolgus monkeys. The results resemble those obtained with rhesus testicular tissue. It is possible that bladder tissue could be used in roller tubes with the same success that

27. Shwartzman, G. 1950, Proc. Soc. Exper. Biol. & Med. **75**: 835–838.

has been obtained with rhesus testicular tissue.

Cynomolgus ileum.—The tissue used in tests of cynomolgus ileum was largely from the muscular coats, inasmuch as most of the mucosa was scraped off in order to reduce contamination. Hof. virus multiplied vigorously through 3 passages in this tissue. A specimen of fluid removed from the 3rd passage at 11 days and diluted 100-fold infected all of 3 test monkeys. When diluted 1000-fold it infected 2 of 3 test monkeys. The latter dilution represented a 10^{-12} dilution of the original inoculum. No attempt was made to carry this virus through further passages.

Human endometrium.—In one experiment Hof. virus was added to a culture of endometrium from a woman 48 years old. Fluid representing a calculated dilution of 10^{-7} of the inoculated virus caused paralysis of all of 3 injected monkeys. In a second experiment in which tissue from another patient was employed, Lansing virus disappeared almost completely in 6 days at a calculated dilution of 10^{-4}. Hof. virus was also employed with cultures of this tissue and appeared to drop considerably in titer, as only 1 of 3 monkeys was infected with fluid removed at 9 days. Therefore, in one experiment Hof. virus multiplied and in a second experiment neither Hof. nor Lansing virus multiplied.

Cell strains of mouse, rat and human origin, adapted to in vitro growth prior to inoculation with virus

Cell strains that have been thoroughly adapted to in vitro multiplication often change their biological characteristics. They may multiply more rapidly and in other ways appear to be more undifferentiated than in the normal tissues from which they were derived. It appeared desirable to determine whether cell strains of this sort were susceptible to poliomyelitis virus. For these studies 3 cell lines, derived from 3 different animals of origin, rat, mouse and man, were chosen. For comparative purposes 2 cell lines cultivated in our laboratory from human embryonic tissue were similarly studied. Essential data concerning the several cell strains are given in table 3.

Roller tube cultures of Gey's cell strain D_1Re, derived from a human chondromyxosarcoma about 1934,[28] were inoculated with Mahoney virus as a 2% suspension of infected CNS. No degeneration of cells occurred during a 17-day observation period. No virus was detected when fluid removed at 10 days, which represented a calculated dilution of about 10^{-5}, was injected into monkeys. In a second experiment with Mahoney virus and in one with Hof. virus, similar results were obtained; there was no increase of virus and no detectable cytonecrosis. Therefore, it was concluded that the D_1Re strain of cells had failed to support the growth of Mahoney or of Hof. poliomyelitis virus.

Gey's cell strain T-333, rat fibroblasts which became malignant in tissue culture,[24] was found to be resistant to infection with Hof. and Y-SK poliomyelitis viruses. No degeneration of cells and no multiplication of virus occurred in 3 experiments with these 2 virus strains.

Cultures of Earle's L strain of mouse cells[23] were inoculated with Mahoney and with Y-SK viruses, both after 3 passages in monkey testicular tissue. Neither virus multiplied or caused degeneration of these cells. Absence of multiplication of virus was based on

---

28. Gey, G. O., Gey, M. K., Inui, F. and Vedder, H. 1945, Bull. Johns Hopkins Hosp. **77**: 116–131.

TABLE 3.—*Results of tests for multiplication of poliomyelitis virus in stock strains of fibroblasts.*

Strain designation	Species and tissue of origin	Age	Multiplication of poliomyelitis virus
D_1Re (Gey)	Human chondromyxosarcoma	17 years	−
T-333 (Gey)	Rat fibroblasts	13 years	−
L-strain (Earle)	Mouse subcutaneous tissue	12 years	−
X-401 (Univ. of Wash.)	Human embryonic tissue	1 month	+
X-243 (Univ. of Wash.)	Human embryonic tissue	2½ months	+

negative results of animal inoculation of tissue culture fluids harvested at intervals ranging from 7 to 15 days.

Strain D_1Re and T-333 have supported growth of Eastern equine encephalomyelitis virus in experiments reported by Bang and Gey.[29] As a check on the techniques employed in experiments in our laboratory, cultures of L strain cells were inoculated with WEE virus. The virus grew extensively. WEE virus and MM virus both caused widespread degeneration of cells in cultures of T-333. Although MM virus multiplied extensively on this tissue, WEE virus was not carried in subcultures far enough to demonstrate multiplication.

The strains of human embryonic tissue started in our laboratory were tested with Y-SK and Hof. strains of poliomyelitis virus. Both strains of virus grew well and caused prompt and severe necrosis of cells of both strains. MM virus also grew well and caused extensive and rapid degeneration of the one strain in which it was tested.

DISCUSSION

It seems possible that the resistance of mice and rhesus monkeys to orally inoculated poliomyelitis virus may be related in some way to a low susceptibility of extraneural tissues of these animals to the virus. It may be more than coincidence that poliomyelitis virus multiplied most readily in tissue from cynomolgus monkeys and that this species is susceptible to oral infection with the virus.

It is noteworthy that poliomyelitis virus grew abundantly in only 1 of 7 experiments with rhesus testicular tissue in flask cultures. In contrast, the virus multiplied regularly and abundantly in roller tube cultures of such tissue, with a good outgrowth of susceptible cells like fibroblasts. The relatively low nutrient content of our flask medium did not favor the outgrowth of cells. It is not clear whether the significant change that occurred in roller tubes, but not in most flask cultures of rhesus testicular tissues, was quantitative and due to an increase in the number of susceptible cells, or was qualitative and represented a change of some cell type from resistance to susceptibility.

In view of the results obtained with rhesus testicular tissue, it appears clear that future tests of any tissue for its capacity to support the growth of poliomyelitis virus should be carried out with a tissue culture system, in which the cells under consideration are able to multiply. A simple maintenance medium, of the sort commonly employed with suspended cell cultures, may not be adequate. In fact, it is possible that the successful growth of poliomyelitis virus in suspended cell cultures of human embryonic tissue and human and cynomolgus testicular tissue may result in part from enrichment of the medium through degeneration of the embryonic cells or germinal cells.

The recent success of Scherer, Syver-

29. Bang, F. B. and Gey, G. O. 1951, Tr. New York Acad. Sc. Ser. II: **13**: 324–327.

ton and Gey[30] in cultivating poliomyelitis viruses in a stock strain of cells of a human epidermoid carcinoma of the uterine cervix represents the achievement of an important objective. It seems desirable, however, to point out some advantages in using tissue cultures that have been maintained for a comparatively short time in vitro. The changes which occur in the cells of a stock culture over a period of several years may be considerable. This is analogous to the well known changes that occur in laboratory strains of microorganisms. Such changes make cultures of many fungi completely unidentifiable after relatively short periods of in vitro cultivation. In view of the changes that may be expected to occur in stock tissue cultures, it is quite possible that experimental results of investigators working in different laboratories and many years apart, can be more reliably compared if tissue cultures are made from fresh tissue and maintained under carefully specified conditions for relatively short periods of time than if stock cultures several years old are used.

SUMMARY AND CONCLUSIONS

A survey was conducted to determine the capacity of poliomyelitis virus to multiply in tissue cultures of tissues from various laboratory and domestic animals. With a few exceptions, the tissue cultures were of the suspended cell type and were prepared with a fluid medium of low nutrient value (serum ultrafiltrate and balanced salt solution). Type 1 (Lansing and Y-SK) and type 2 (Hof. and Mahoney) strains of virus were employed.

In either adult or immature testicular tissue from cynomolgus monkeys, active multiplication of virus occurred regularly. The minimal infective dose of virus was essentially the same as that for intracranially injected monkeys. The amount of virus produced in tissue culture, using infected CNS tissue as inoculum, was approximately equal to the amount contained in a comparable amount of spinal cord tissue from an infected animal.

Explants of cynomolgus testicular tissue removed from an infected culture usually exhibited a good outgrowth of fibroblasts if transferred to plasma clot cultures with a more enriched medium. It was not possible to distinguish infected from uninfected cultures by direct examination, or by examination of explants transferred in this manner.

Rhesus testicular tissue in flask cultures failed to support growth of virus in four experiments, and gave a low level of multiplication in two experiments. Virus multiplied extensively in a single experiment in which tissue came from a mature animal exposed to roentgen irradiation of the testes.

Poliomyelitis virus multiplied in cynomolgus intestinal tissue from which the mucosa had been largely removed, and less extensively in urinary bladder tissue. It did not multiply in cardiac or skeletal muscle.

No multiplication of virus occurred in testicular tissue from mice, hamsters, guinea pigs, rabbits, dogs and bulls, nor in urinary bladder tissue from mice. Irradiation of mice with X rays and injection of hamsters with cortisone failed to render their testicular tissue capable of supporting in vitro virus multiplication. Virus multiplied in human endometrial tissue in one of two experiments.

No multiplication of virus occurred in three different strains of fibroblasts that had been maintained in vitro many years. These were Earle's L strain, derived from mouse tissue, Gey's D_1Re strain, which originated in a

30. Scherer, W. F., Syverton, J. T. and Gey, G. O. 1953, J. Exper. Med. **97**: 695–710.

human sarcoma, and Gey's T-333 strain, from rat tissue. Virus multiplication occurred in human embryonic tissue maintained for 1 to 2 months in roller tubes with a medium favorable for fibroblast multiplication.

In view of the poor results obtained with rhesus testicular tissue in flask cultures, and the regular and abundant multiplication when this tissue is maintained in roller tubes with a medium favorable for fibroblast proliferation, it is concluded that our inability to obtain multiplication with poliomyelitis virus in tissue cultures of skeletal and cardiac muscle of monkeys, and testicular tissue of animals other than monkeys and man, is not good evidence for the complete resistance of these tissues. Further studies on these and other tissues should be made, using roller tubes or some comparable system before a firm decision is reached concerning their susceptibliity or resistance to poliomyelitis virus.

SPORADIC BOVINE ENCEPHALOMYELITIS

3. REPRODUCTION OF THE DISEASE, WITH PARTICULAR REFERENCE TO THE ROLE OF POLIOMYELITIS VIRUSES IN EXPERIMENTAL INFECTION IN CALVES

HERBERT A. WENNER, ROBERT W. MENGES, AND GERALD S. HARSHFIELD*

From the Hixon Memorial Laboratory, University of Kansas School of Medicine, Kansas City, Kansas; the Communicable Disease Center, Public Health Service, Department of Health, Education, and Welfare, Kansas City, Kansas; and the South Dakota State College, Brookings, South Dakota

In preceding papers[1,2] we reported on the natural history of sporadic bovine encephalomyelitis. Recognition was given to the clinical and pathologic changes observed, as well as to certain aspects of epizootiology among affected cattle. In studies on etiology nine strains of an infectious agent were detected in tissues obtained from infected calves. In each instance the agent was found to be a member of the psittacosis-lymphogranuloma family of viruses.

Following identification of the agent isolated from calves as a psittacosis-like virus, a series of experiments were made in order to define whether the clinical syndrome known as bovine encephalomyelitis (BE) could be reproduced in calves. The simplicity of these experiments was compounded by a previous report[3] that an agent related to the Lansing strain (type 2) of poliomyelitis virus had been found in yolk sac of embryonated eggs, previously inoculated with BE virus. It therefore became necessary to determine a relationship, if any existed, between the Lansing-like (BE-L) virus and sporadic bovine encephalomyelitis.

The studies reported here have been divided into two parts. The first section is concerned with infection produced in experimental calves with BE viruses. The second section is concerned with the position of poliomyelitis viruses in the pathogenesis of sporadic bovine encephalomyelitis.

MATERIALS AND METHODS

Viruses. BE virus.—Three strains were used: W.S., C.L., and J.W. The histories of these strains have been described.[1,2] *BE-L virus.*—Two strains, W.S.-L. and C.L.-L., were obtained by inoculation of mice with yolk sac emulsion obtained from infected chick embryos. Each of these strains was serologically related to the Lansing strain (type 2) of poliomyelitis virus.[3] *Poliomyelitis virus.*—Three strains were employed, namely, Lansing (type 2), Fotovich (type 2), and Pos (type 1). A history of these strains has been published.[4] A brief synopsis of

Received for publication September 23, 1953.

Approved for publication by the South Dakota Experimental Station as Journal Series no. 309.

Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

* With the technical assistance of Jeannette S. Carter and Paul Kamitsuka.

1. Menges, R. W., Harshfield, G. S. and Wenner, H. A. 1953, Sporadic bovine encephalomyelitis. 1. The natural history of the disease in cattle. Am. J. Hyg. **57**: 1–14.
2. Wenner, H. A., Harshfield, G. S., Chang, T. W. and Menges, R. W. 1953, Sporadic bovine encephalomyelitis. 2. Studies on the etiology of the disease. Isolation of nine strains of an infectious agent from naturally infected cattle. Am. J. Hyg. **57**: 15–29.
3. Chang, T. W. and Wenner, H. A. 1951, Interrelationships observed between the Lansing strain of poliomyelitis virus and the virus of sporadic bovine encephalomyelitis. Proc. Soc. Exper. Biol. & Med. **78**: 659–662.
4. The Committee on Typing. 1951, Immunologic classification of poliomyelitis viruses. 1.

TABLE 1.—*Historical summary of viruses employed in the experimental studies.*

Strain	Source	Year of isolation	Host susceptibility*						Number of passages
			Chick embryos	Mice	Monkeys	Cotton rats	Hamsters	Guinea pigs	
Bovine encephalomyelitis viruses (psittacine)									
W.S.	Calf CNS	1949	+	+	+	+	+	+	36 (YS)
C.L.	Calf liver and spleen	1949	+	+	+	+	+	+	40 (YS)
J.W.	Calf NS	1952	+	0	±	±	+	+	5 (YS)
BE-L viruses, strains serologically related to Lansing virus									
W.S.-L.	Infected yolk sac	1950	0	+	+	+	+	+**	4 (M)
C.L.-L.	Infected yolk sac	1950	0	+	+	+	0	+**	4 (Rh)
Poliomyelitis viruses									
Lansing	Human cord	1937	0	+	+	+	ND	ND	300? (M)
Fotovich	Human cord	1948	0	+	+	+	ND	ND	4 (Rh)
Pos	Human stool	1941	0	0	+	0	ND	ND	8? (Rh)

* Chick embryos were inoculated by way of the yolk sac; guinea pigs by way of intracerebral or intraperitoneal portals; in all others the intracerebral portal was used.
** Encephalitis, only.
Rh = monkeys.
YS = yolk sac.
ND = not done.
M = mice.
CNS = central nervous system.
Guinea pigs weighed 500 to 600 g.

the history of the viruses included in the study appears in table 1.

The experimental area.—The Department of Veterinary Science at the South Dakota Agricultural Experimental Station maintains a barn designed for experimental veterinary medicine (fig. 1). In the forepart of the structure are an autopsy room, a preparation room, and two laboratories. In the rear there are 8 stalls. Each stall is designed as a separate isolation unit. Partitions between stalls are 5 feet high. Feed, urine, or feces cannot inadvertently enter another stall.

Inoculations were done in the autopsy room. As a rule, two calves occupied a stall. Calves occupying the same stall received the same inoculum by the same route. Observations on calves were made twice daily; the temperature was recorded once daily. At indicated intervals, blood, serums, stools, and nasal discharges were obtained from calves; these were placed in sterile containers and stored in an electric refrigerator at −20 C.

Calves.—Sixteen calves, ranging in age from 2 weeks to 8 months, were used in the experiments. All were dairy-type males, born and reared on the experimental farm located at South Dakota State College, Brookings, South Dakota. The history of each calf used in the experimental studies appears in table 2.

Whole blood, serum, and feces were obtained from each calf prior to inoculation. Inoculation of calves was made through intracerebral, intraperitoneal, or subcutaneous portals. For intracerebral inoculation the calves were restrained by a rope hitch while lying on their sides in the animal operating room. Under light anaesthesia (nembutal) a trephined opening was made approximately 1 inch above the eye and just lateral to the midline. Using a 3-inch needle, the inoculum was directed inward and downward into the area of the thalami. Selection of the inoculation side was determined by trials on the heads of slaughtered animals. Intraperitoneal inoculations were made through the right flank below the transverse processes of the lumbar vertebrae. Subcutaneous inoculations were made in the skin of the neck. The skin sites were prepared for inoculation by clipping, shaving, and applying iodine and alcohol. Instruments were sterilized by autoclaving or boiling.

At the end of the experiment, twelve of the calves were killed. The visceral organs, brain, and spinal cord were carefully examined. Appropriate tissues were obtained for histologic examinations.

Preparation of specimens for detection of viruses.—Whole blood, feces, and nasopharyngeal exudates were studied in mice, guinea pigs, monkeys, and in tissue culture. Guinea pigs were employed for the detection of BE virus. Mice, monkeys, and tissue cultures of monkey kidney epithelium were used for the detection of poliomyelitis viruses. Materials inoculated into guinea pigs were used in aqueous suspensions. Stool extracts, prior to intracerebral inoculation of mice and monkeys, were treated with ether.[5] Nasopharyngeal exudates were eluted[6] from cotton pledgets

---

A cooperative program for the typing of one hundred strains. Am. J. Hyg. **54**: 201–203.

TABLE 2.—*History of calves* used in the experimental study.*

Calf no.	Breed	Date of birth	Status of first inoculation		Status of immunity to‡	
			Age	Weight (lb.)†	BEV	Type 2 polio virus
102	Jersey	1/30/52	2 wk.	75	None	None
103	Holstein	11/19/51	3 mo.	200	None	None
104	Holstein	1/16/52	4 wk.	125	None	None
106	Holstein	12/13/51	2 mo.	150	None	None
107	Guernsey	1/ 3/52	6 wk.	120	None	None
108	Guernsey	11/19/51	3 mo.	150	None	None
109	Brown Swiss	11/ 5/51	3 mo.	200	None	None
111	Brown Swiss	6/ 3/52	5 mo.	275	None	None
112	Holstein	9/25/52	6 wk.	140	None	None
113	Holstein	4/24/52	6 mo.	375	None	1:4
114	Guernsey	4/ 9/52	6½ mo.	400	?Immune	1:4
115	Holstein	4/ 8/52	6½ mo.	400	?Immune	1:4
116	Holstein	7/18/52	4½ mo.	175	None	None
117	Guernsey	8/12/52	3 mo.	125	None	None
118	Holstein	3/ 7/52	8 mo.	475	None	1:32
119	Holstein	3/26/52	7½ mo.	400	None	1:8

The calves were from the South Dakota State College Dairy. All were males, and were weaned In the 4th day. Calves received dam's milk for 4 days; fed pooled herd milk for a week following.

* No history of BE infection in the herd at any time.

† All weights are estimated.

‡ CF antibodies absent in serum dilution 1:4 (BEV). Neutralizing antibodies absent in serum dilution 1:4 (type 2 poliomyelitis virus).

prior to inoculation of animals or tissue cultures.

Criteria for establishing infection.—The criteria used for establishing infection with the psittacosis-lymphogranuloma venereum group and the poliomyelitis group of viruses have been described.[5,7,8]

Briefly outlined, animals sacrificed because of clinical disease were studied in regard to (a) antibody response, (b) the presence or absence of elementary bodies, and (c) histologic changes observed in experimental infection with BE and poliomyelitis viruses. The presence of a cytopathogenic agent in tissue cultures was specifically studied with type-specific poliomyelitis antiserums in order to establish its identity. Passage was made of tissue extracts in yolk sacs of embryonated eggs, in experimental animals, and in tissue culture. Impression smears were stained by Macchiavello's method. Giemsa's method was used to stain tissue specimens.

Serologic tests.—Serums were obtained from calves, monkeys, and guinea pigs, before and periodically after inoculation. Neutralization and complement-fixation (CF) tests were employed.

The 50% neutralization end point against 32PD_{50} of Lansing (type 2) poliomyelitis virus was determined in mice, using 4-fold serum dilutions. The pool of virus used had a PD_{50} of $10^{-6.9}$. A virus titration was included in each test.

The following antigens were used in CF tests: W.S. and J.W. strains of BE virus, Lygranum* Brunhilde (type 1), and Y-SK (type 2) poliomyelitis viruses.† Box titrations were done to determine optimal antigenic activity of each antigen. Four to eight units of antigen were used. The method used in CF tests have been described.[9,10] Confirmatory and supplementary

5. Trask, J. D., Vignec, A. J. and Paul, J. R. 1938, Isolation of poliomyelitis virus from human stools. Proc. Soc. Exper. Biol. & Med. **38:** 147–149.
6. Wenner, H. A. 1945, Effect of H concentration on eluting poliomyelitis virus from cotton fiber. Proc. Soc. Exper. Biol. & Med. **60:** 104–106.
7. Enders, J. F., Weller, T. H. and Robbins, F. C. 1949, Cultivation of the Lansing strain of poliomyelitis virus in cultures of various embryonic tissues. Science, **109:** 85–87; Riordan, J. T., Ledinko, N. and Melnick, J. L. 1952, Multiplication of poliomyelitis viruses in tissue cultures of monkey testes. 2. Direct isolation and typing of strains from human stools and spinal cords in roller tubes. Am. J. Hyg. **55:** 339–346.
8. Meyer, K. F. and Eddie, B. 1948, Psittacosis. Diagnostic Procedures for Virus and Rickettsial Diseases, New York, Publications Office Am. Pub. Health Assoc.

* Acknowledgment is made E. R. Squibb and Sons, New Brunswick, New Jersey, for supplying us with lymphogranuloma venereum (Lygranum) antigen.

† Type-specific poliomyelitis virus antigens were prepared according to the method of Svedmyr, Enders, and Holloway.[9]

9. Svedmyr, A., Enders, J. F. and Holloway, A. 1953, Complement fixation with the three types of poliomyelitis viruses propagated in tissue culture. Am. J. Hyg. **57:** 60–70.
10. Wenner, H. A., Monley, A. and Todd, R. N.

tests were done in the National Microbiological Institute, National Institutes of Health, Hamilton, Montana‡ with the following antigens: BE, W.S., Q fever, Rocky Mountain spotted fever, psittacosis, ornithosis, and endemic typhus.

PART I

EXPERIMENTAL INFECTION OF CALVES WITH BOVINE ENCEPHALOMYELITIS VIRUSES

Clinical course.—Five calves were inoculated with two strains of BE viruses. These agents were morphologically and serologically related to the psittacosis-lymphogranuloma group of agents. Intracerebral, subcutaneous, and intranasal portals were used. The calf inoculated intranasally had no objective symptoms, and ostensibly it remained well. None of the other calves experienced anything more than a mild illness. Indeed, had not daily temperature recordings been made, the illness might have been missed in three calves. Recovery occurred in all; clinical signs of encephalitis were not seen in any of the calves.

The febrile episodes were biphasic. An initial fever (103 to 105 F, rectal) lasting 24 to 48 hours occurred on the 2nd and 3rd day after inoculation. The temperature then became subnormal for a period of 3 to 7 days. A second bout of fever (104 to 105 F, rectal) occurred for a period of 7 to 10 days, beginning in the 2nd week after inoculation with BE virus. During the period when the temperature was subnormal there was an observed leukopenia; however, the differential leukocyte pattern was within normal limits. Clinical and laboratory data obtained in studies of two calves appear in figures 2 and 3. A summary of the observations made during the experimental studies of the five calves inoculated with BE viruses appears in table 3.

Autopsy findings.—Three calves were sacrificed 4 to 6 weeks after inoculation with BE virus. The lesions in each were similar to those found in calves autopsied in the field during natural outbreaks of sporadic bovine encephalomyelitis. There was organized exudate, or exudate undergoing organization, involving peritoneal, pleural, and pericardial membranes. The gross findings were confirmed by histologic examination of visceral organs. Encephalitis such as that described[1] in calves succumbing as a result of natural infection was not seen. In scattered foci, perivascular cuffs were found in two of the three animals examined. There was infiltration of meningeal veins with mononuclear cells. Calf 103 apparently experienced a vascular reaction following challenge inoculation with a heterotypic strain of BE virus. The animal had a large number of circulating eosinophils, and a marked infiltration of eosinophilic cells in perivascular spaces of the dura mater.

Antibody response.—Serum antibodies appeared following infection with BE viruses. Complement-fixing antibodies for BE antigen appeared in three of the five experimental calves. Serum antibodies apparently failed to develop in calf 102, despite the presence of gross infection at necropsy. Antibodies against psittacosis and Lygranum antigens appeared in the serum of calf 119; antibodies were not found with BE antigens. Calf 119 resisted infection on challenge 2 months later with the J.W. strain of BE virus. The observed serum antibody levels in experimental calves are recorded in table 3. Additional and

1950, Studies on complement fixation with Newcastle disease virus. J. Immunol. **64**: 323–333.

‡ Acknowledgment is also given R. K. Gerloft and D. B. Lackman for valued assistance in serologic studies. The tests were performed at the National Microbiological Institute, National Institutes of Health, Hamilton, Montana.

TABLE 3.—*Results of tests made in calves with two strains of BE virus.*

Calf no.	Inoculum: Date	Source yolk sac	Amount in ml	Portal	Clinical signs: Fever	Weight loss	Depression	Diarrhea	Ataxia	Encephalitis	Antibody tests: Complement fixation: Bovine encephalitis	Lymphogranuloma venereum (Lygranum)	Psittacosis	Q fever	Rocky Mountain spotted fever	Brunhilde (type 1) poliomyelitis	Lansing (type 2) poliomyelitis	N: Lansing (type 2) poliomyelitis	Pathology: Gross: Peritoneal exudate	Pleuritis	Pericarditis	Serositis	Microscopic: Encephalitis
102	2/14/52	W.S.	1.0	IC	++++	0	+	0	0	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/4	++	++	++	++	+*
103	2/14/52	W.S.	1.0	IC	++++	+	+	0	+	0	0/48	0/16	0/16	0/8	0/16	0/0	0/0	<8/<2	ND	ND	ND	ND	ND
111	11/ 5/52	W.S.	6.0	SC	0	0	0	0	0	0	0/64	0/32	0/32	0/0	0/0	0/0	16/16	2/<2	+	+	+	ND	0
114	1/ 2/53	J.W.	4.0	SC	+	+	+	0	0	0	4/256	16/64	4/768	0/16	4/0	0/0	0/0	4/<2	ND	ND	ND	ND	ND
119	11/ 5/52	W.S.	20.0	IN	0	0	0	0	0	0	0/0	4/32	0/12	0/0	0/0	0/0	0/0	8/8	ND	ND	ND	ND	ND

0/32 = negative preinoculation/serum titer 1:32, 30 to 60 days later.
N = neutralization, 50% serum dilution end point. IC = intracerebral. IP = intraperitoneal. SC = subcutaneous. IN = intranasal.
* Perivascular infiltration present in dura mater.

supporting data on six additional calves inoculated with BE viruses appear in table 5.

Virus isolations.—A study limited to the detection of virus in blood, stool, and nasopharyngeal exudates was done on specimens obtained between the 2nd and 4th weeks after inoculation with BE viruses. BE virus was detected in the blood obtained from 3 calves during the second bout of fever, which occurred 8 to 14 days after inoculation with BE virus. BE virus was not detected in feces obtained from 5 calves, or in nasopharyngeal exudates obtained from 4 calves. Unfortunately, a diet containing aureomycin was fed guinea pigs during part of the experimental period, probably thereby obscuring infection and rendering doubtful the validity of these tests.

Summary.—Two strains of BE viruses, originally obtained from cattle sick of encephalomyelitis, were employed in experimental infection of calves. A third strain, employed later in challenge experiments, gave corresponding results. A mild clinical illness occurred, attended by the presence of visceral exudates characteristically found in the naturally-occurring disease, and followed by the appearance of group-specific antibodies for the psittacosis-lymphogranuloma venereum family of viruses. Frank clinical signs of encephalomyelitis were not observed. Focal perivascular infiltration in meningeal veins were present in four experimental calves (including one calf appearing in table 5). These latter findings are in sharp contrast with diffuse encephalomyelitis encountered among animals succumbing during epizootics. Antibodies against BE viruses developed during the postinoculation period. Antibodies against type 2 poliomyelitis virus were not produced following the natural disease in calves. In three instances BE virus was recovered

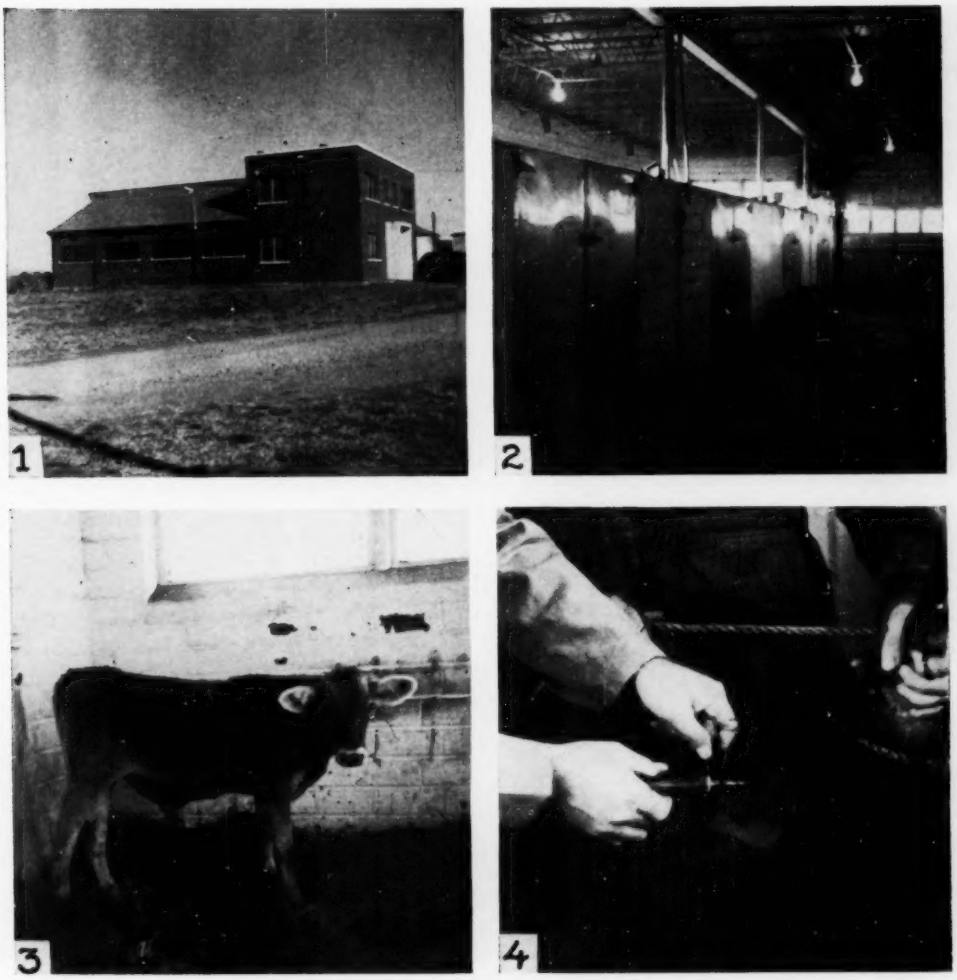


FIG. 1.—1. The experimental barn. 2. Entry to 4 isolation stalls from the runway. 3. An isolation stall, calf 109. 4. Inoculating a calf subcutaneously with BE virus.

from the blood of calves obtained during the acute phase of illness.

PART II

THE ROLE OF POLIOMYELITIS VIRUSES IN THE PATHOGENESIS OF BOVINE ENCEPHALOMYELITIS

In an earlier report[3] Chang and Wenner set aside two strains of bovine encephalomyelitis viruses as being immunologically related to the Lansing strain of poliomyelitis virus. These strains, adapted to mice, monkeys, and hamsters, produced flaccid paralysis and, in addition, histologic lesions characteristically seen in experimental poliomyelitis. In order to determine the position of type 2 strains of poliomyelitis viruses in bovine encephalitis, further experimental studies were made in calves. These studies have an additional interest in view of the occurrence of type 2 antibodies in serums heretofore obtained from cattle.[11]

11. Hammon, W. M., Mack, W. N. and Reeves, W. C. 1947, The significance of protection tests with the serum of man and other animals against the Lansing strain of poliomyelitis virus. J. Immunol. **57**: 285–299.

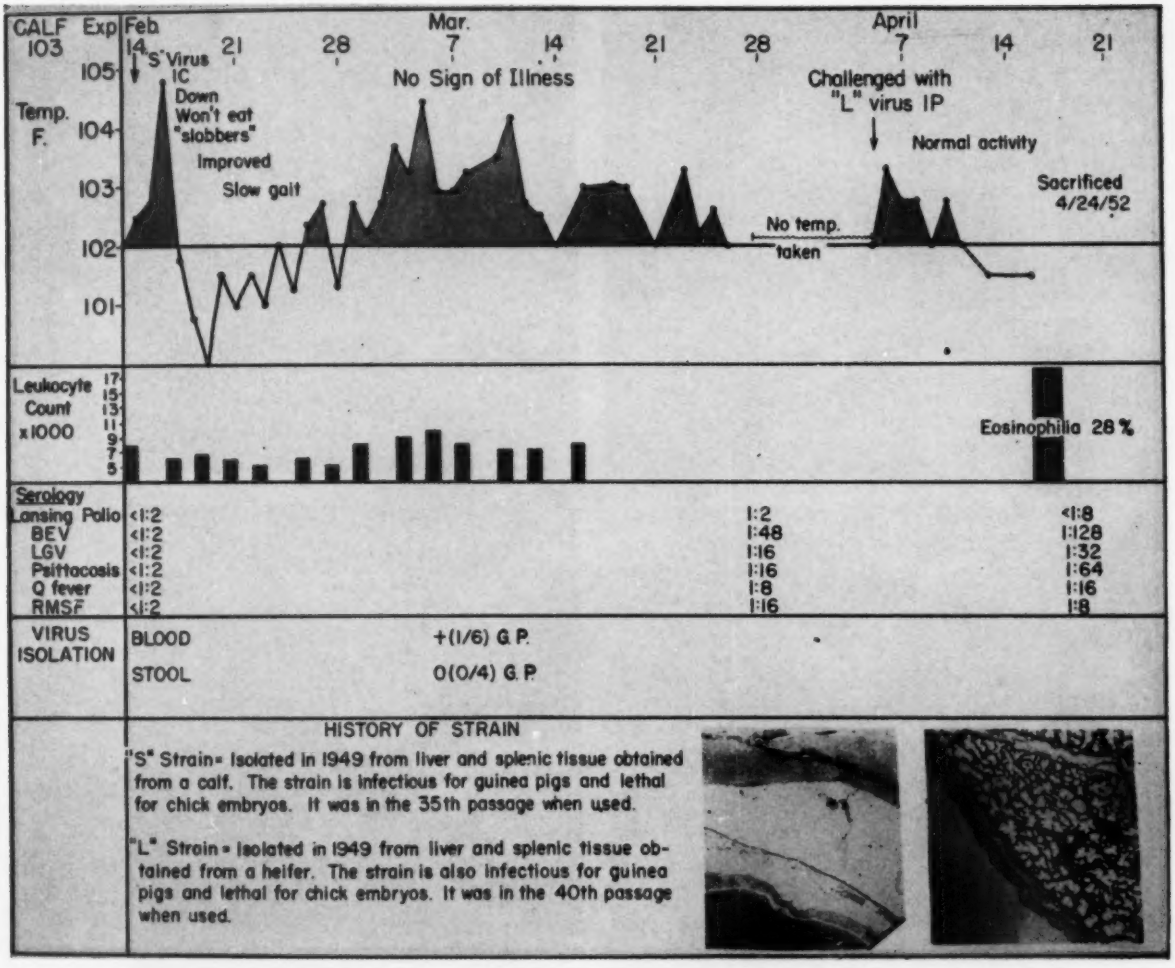


FIG. 2.—Summary of clinical and laboratory data following inoculation of calf 103 with the W.S. strain of BE virus. The animal was challenged with the C.L. strain of BE virus 52 days later. Microphotographs of intense eosinophilic perivascular reaction in blood vessels of the dura and organized fibrous pleuritis appear in the lower right corner of the figure.

The two strains of viruses detected in the yolk sac of embryos infected with the W.S. and C.L. strains of BE virus have been described.[3] In addition, these strains have been adapted to monkey kidney epithelial cells in tissue culture. The cytopathogenic effect produced by each was inhibited by type 2 (Lansing) poliomyelitis antiserum.

In order to ascertain whether these two viruses could cause apparent or inapparent illness, four calves were inoculated intracerebrally with virus obtained from paralyzed monkeys (4th passage) and mice (4th passage). Five additional calves were inoculated with two other strains of type 2 poliomyelitis viruses. The results of these tests appear in table 4. The results were entirely negative. No obvious clinical illness occurred. Two calves had fever. Calf 117 had a rise in temperature to 104.4 F on the 13th day; calf 118 had fever ranging between 102.8 and 104.5 F during the 2nd week following inoculation with the Fotovich strain of virus.

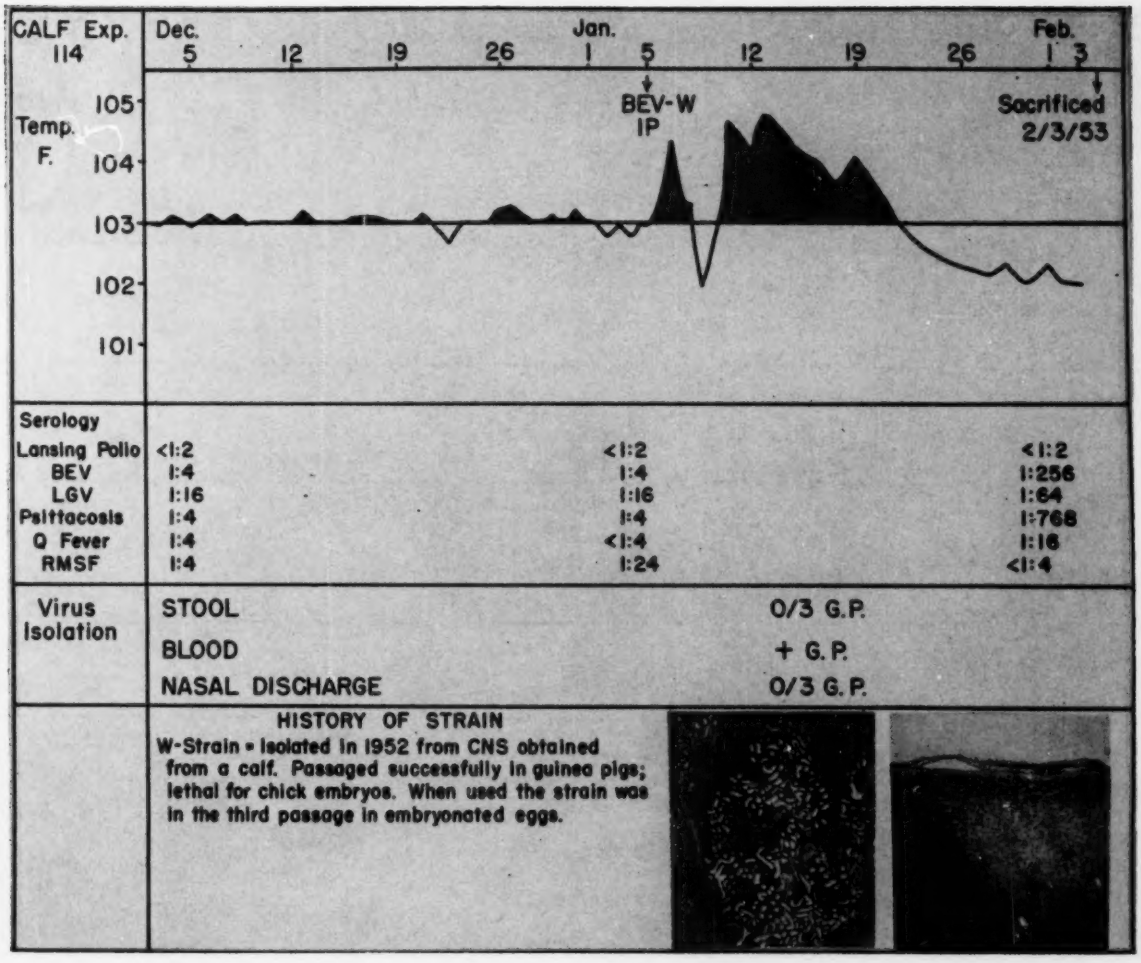

CALF Exp. 114
Dec. 5 12 19 26
Jan. 1 5 12 19 26
Feb. 1 3
Temp. F.
105
104
103
102
101
BEV-W IP
Sacrificed 2/3/53
Serology
Lansing Polio <1:2 <1:2 <1:2
BEV 1:4 1:4 1:256
LGV 1:16 1:16 1:64
Psittacosis 1:4 1:4 1:768
Q Fever 1:4 <1:4 1:16
RMSF 1:4 1:24 <1:4
Virus Isolation
STOOL 0/3 G.P.
BLOOD + G.P.
NASAL DISCHARGE 0/3 G.P.
HISTORY OF STRAIN
W-Strain • Isolated in 1952 from CNS obtained from a calf. Passaged successfully in guinea pigs; lethal for chick embryos. When used the strain was in the third passage in embryonated eggs.

FIG. 3.—Summary of clinical and laboratory data following inoculation of calf 114 with the J.W. strain of BE virus. The animal had been inoculated November 5, 1952 with normal yolk sac suspension. Microphotographs of granulomatous lesions in the kidney and exudate overlying the liver appear in the lower right corner of the figure.

Autopsy findings.—Three calves were sacrificed in the 7th week after inoculation. The visceral lesions characteristic of infection with BE viruses[1] were not seen. A mild meningoencephalomyelitis was found in calf 104. Otherwise, abnormal histologic changes were not found.

Antibody response.—None of the nine calves developed serum antibodies against type 2 poliomyelitis virus. A rise in titer against members of the psittacosis-lymphogranuloma venereum viruses was observed in serums obtained from three calves. However, the three calves were later challenged, among others, with BE virus. They were not immune (table 5).

Virus isolations.—Blood samples and stool specimens obtained from eight calves inoculated with type 2 poliomyelitis viruses were tested in mice and monkeys. None of the mice or monkeys developed experimental poliomyelitis. A further study of these specimens in tissue culture[7] is being done. Several cyto-

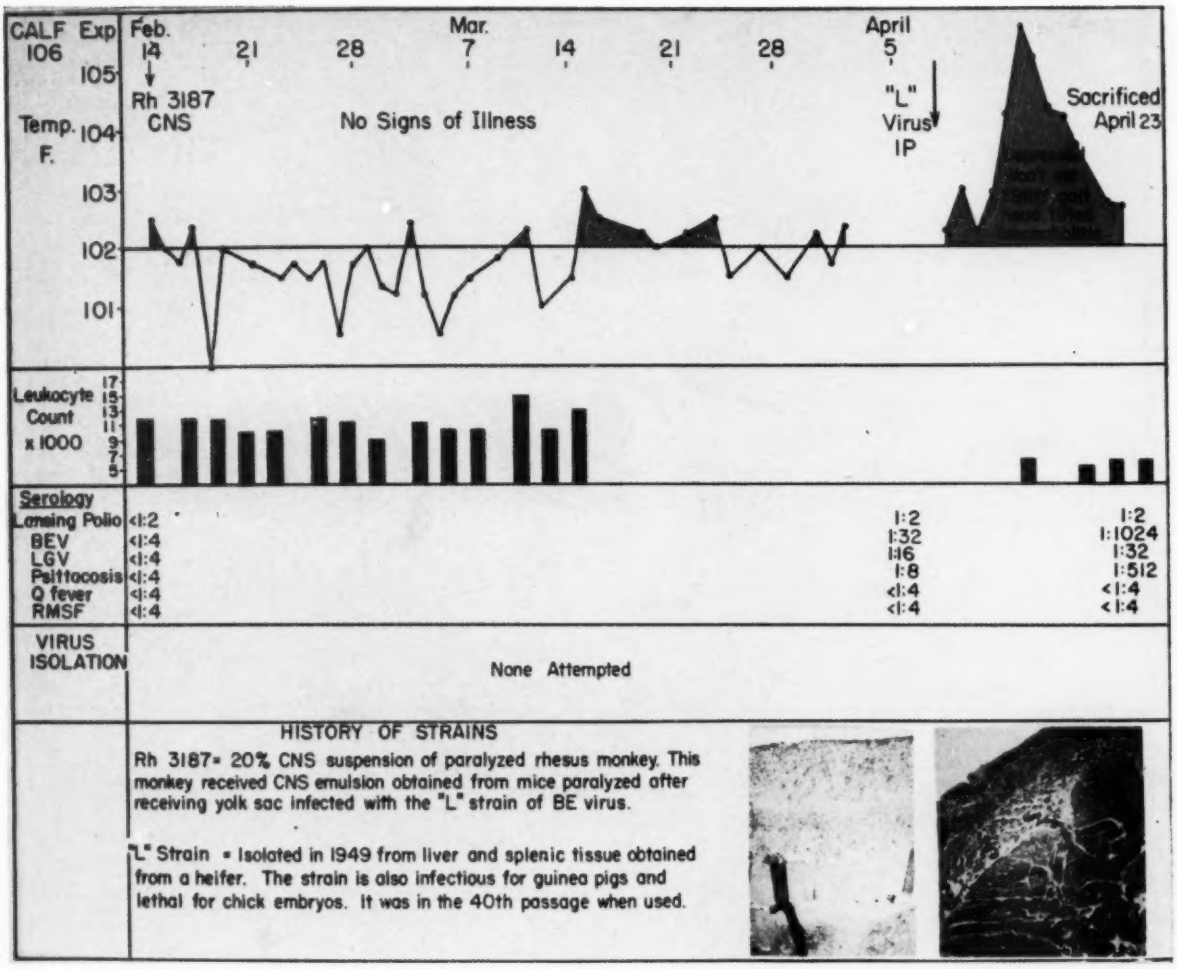

CALF Exp 106
Feb. 14 21 28
Mar. 7 14 21 28
April 5
Temp. F. 105 104 103 102 101
Rh 3187 CNS
No Signs of Illness
"L" Virus IP
Sacrificed April 23
Leukocyte Count x 1000 17 15 13 11 9 7 5
Serology
Lansing Polio <1:2 1:2 1:2
BEV <1:4 1:32 1:1024
LGV <1:4 1:16 1:32
Psittacosis <1:4 1:8 1:512
Q fever <1:4 <1:4 <1:4
RMSF <1:4 <1:4 <1:4
VIRUS ISOLATION
None Attempted
HISTORY OF STRAINS
Rh 3187- 20% CNS suspension of paralyzed rhesus monkey. This monkey received CNS emulsion obtained from mice paralyzed after receiving yolk sac infected with the "L" strain of BE virus.
"L" Strain - Isolated in 1949 from liver and splenic tissue obtained from a heifer. The strain is also infectious for guinea pigs and lethal for chick embryos. It was in the 40th passage when used.

FIG. 4.—Summary of clinical and laboratory data following inoculation of calf 106 with BE-L (Rh 3187 CNS) virus. The animal was challenged with C.L. strain of BE virus on April 5. Microphotographs of exudate overlying the spleen and epicardium appear in the lower right corner of the figure.

pathogenic agents have been found but none has been identified as poliomyelitis virus.

Challenge with BE virus of calves previously inoculated with type 2 poliomyelitis viruses.—Six of the nine calves inoculated with type 2 poliomyelitis virus were challenged in the 8th week with active strains of BE virus. One calf (118) was observed without challenge inoculation through the 4 months duration of the experimental period. The results obtained in studies made of these calves appear in table 5.

None of the six calves previously inoculated with type 2 viruses was immune to challenge with BE virus. A mild clinical infection was experienced, consisting of fever, weight loss, and physical depression. A consistent rise in serum antibodies was found against BE and related members of the psittacosis-lymphogranuloma group. At necropsy, performed later in convalescence, there was gross evidence of peritonitis, pleurisy, and pericarditis. The gross findings were confirmed by histologic examination. A graphic summary of the clinical

TABLE 4.—*Results of tests made in calves with two strains of virus detected in mice inoculated with BE virus, and immunologically related to type 2 poliomyelitis virus.*

Calf no.	Inoculum				Clinical signs						Antibody tests: Complement fixation							N	Pathology: Gross				Pathology: Microscopic
	Date	Source CNS	Amount in ml	Portal	Fever	Weight loss	Depression	Diarrhea	Ataxia	Encephalitis	Bovine encephalitis	Lymphogranuloma venereum (Lygranum)	Ornithosis	Q fever	Rocky Mountain spotted fever	Brunhilde (type 1) poliomyelitis	Lansing (type 2) poliomyelitis	Lansing (type 2) poliomyelitis	Peritoneal exudate	Pleuritis	Pericarditis	Serositis	Encephalitis
104	2/14/52	Rh 3187	1.0	IC	0	0	0	+	0	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0	0	0	+
106	2/14/52	Rh 3187	1.0	IC	0	0	0	0	0	0	0/32	0/16	0/8	0/0	0/8	0/0	0/0	0/0	Challenged 4/5/52 with BE virus				
107	2/14/52	Mouse	1.0	IC	0	++	0	0	0	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0	0	0	0	0
108	2/14/52	Mouse	1.0	IC	0	0	0	0	0	0	0/16	0/4	0/0	0/0	0/0	0/0	0/0	0/<8	Challenged 4/5/52 with BE virus				
109	2/14/52	Lansing mouse	1.7	IC	0	0	0	0	0	0	0/32	0/4	0/24	0/0	0/0	0/0	0/0	<2/<8	Challenged 4/5/52 with BE virus				
115	11/ 5/52	Fotovich monkey	8.0	IP	0	0	0	0	0	0	32/16	16/16	8/16	0/0	0/0	0/0	0/0	4/<2	Challenged 1/2/53 with BE virus				
116	11/ 5/52	Fotovich monkey	1.0	IC	0	0	0	0	0	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	2/<2	ND	ND	ND	ND	ND
117	11/ 5/52	Fotovich monkey	1.0	IC	+	0	0	0	0	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	<2/2	Challenged 1/2/53 with BE virus				
118	11/ 5/52	Fotovich monkey	20.0	IN	+	+	0	0	0	0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	32/64	0	0	0	0	0

Legend as in table 3.
Rh 3187 = C.L.-L strain of virus, CNS tissue of a paralyzed monkey.
Mouse = W.S.-L strain of virus, CNS tissue of paralyzed mice.

TABLE 5.—*Results of tests made in calves previously inoculated with type 2 poliomyelitis viruses and subsequently challenged with BE virus.*

Calf no.	Challenge inoculum				Clinical signs						Antibody tests: Complement fixation							N	Pathology: Gross				Pathology: Microscopic
	Date	Source yolk sac	Amount in ml	Portal	Fever	Weight loss	Depression	Diarrhea	Ataxia	Encephalitis	Bovine encephalitis	Lymphogranuloma venereum (Lygranum)	Ornithosis	Q fever	Rocky Mountain spotted fever	Brunhilde (type 1) poliomyelitis	Lansing (type 2) poliomyelitis	Lansing (type 2) poliomyelitis	Peritoneal exudate	Pleuritis	Pericarditis	Serositis	Encephalitis
106	2/14/52	C.L.	10.0	IP	+	+	+	0	0	+?	32/256	16/256	8/96	0/0	8/8	0/0	0/0	2/<4	++	++	++	++	+*
108	2/14/52	C.L.	10.0	IP	0	0	0	0	0	0	16/48	4/128	0/16	0/0	0/0	0/0	0/0	<8/<8	+	+	+	+	0
109	2/14/52	W.S.	10.0	IP	+	+	+	0	0	0	32/192	4/128	24/132	0/0	0/0	0/0	0/0	0/0	+	+	+	+	0
115	11/ 5/52	J.W.	5.0	IP	+	0	0	0	0	0	32/384	16/256	16/128	0/0	0/0	0/0	0/16	<2/<2	+	+	+	+	0
117	11/ 5/52	J.W.	5.0	IP	+	+	0	0	0	0	0/96	4/48	0/64	0/0	0/0	0/0	0/0	<2/6	+	+	+	+	0
118		None	—	—	0	0	0	0	0	0	0/4	4/16	0/0	0/0	0/0	0/0	0/0	128/128	0	0	0	0	0

Legend as in table 3.
Calf 118 received type 2 virus (table 4).

and laboratory findings obtained in calf 106 appears in figure 4.

The data obtained in this, and in earlier tests, indicate that type 2 poliomyelitis viruses, or the related BE-L strains, were unable to cause a definitive clinical illness in calves. The serum antibody levels, except in one instance, remained unchanged. Moreover, exposure to these viruses did not interfere with a later challenge inoculation of active BE virus.

Synergistic effect of BE and poliomyelitis viruses.—To include the possibility that BE virus and poliomyelitis viruses might act together in provoking a more severe insult to the central nervous system than could either one alone, four calves* were inoculated with BE virus and poliomyelitis viruses (types 1 and 2). An illness ensued in calves similar to that which followed experimental infection with BE virus. Serum antibodies against poliomyelitis viruses did not appear; conversely, a satisfactory antibody response against BE virus was prompt and the change was quite marked. Three of the four calves were sacrificed on the 30th day. Each had gross evidence of subsiding inflammation of the serous membranes of the abdomen, heart, and lungs. None showed encephalomyelitis.

Summary.—Four strains of type 2 poliomyelitis viruses, two of them ostensibly obtained from embryonated eggs inoculated with BE viruses, failed to cause any demonstrable illness in calves. Neither gross nor microscopic pathologic changes were observed. Serum antibodies were not evoked against poliomyelitis or BE viruses. Poliomyelitis virus was not detected in blood or feces obtained from the experimental calves.

The inoculation of calves with poliomyelitis viruses failed to provide protection against subsequent infection by BE viruses. The simultaneous inoculation of BE and poliomyelitis viruses failed to alter the course of observed experimental infection.

COMMENTS ON UNEXPLAINED SEROLOGIC FINDINGS

In preinoculation serums obtained from two calves, antibodies in titers 1:4 to 1:32 were found against BE, Lygranum, and psittacosis antigens. Serums obtained from three additional calves inoculated with type 2 poliomyelitis viruses showed CF antibodies (from <1:4 to 1:32) against BE antigen (tables 4 and 5). The serum antibody levels encountered in these instances were low compared with titers generally encountered after infection by BE virus. Despite the presence of these antibodies, none of the calves resisted infection with BE virus. In contradistinction, calves previously infected by BE virus were solidly immune to reinfection, despite low titers of antibody, presumably homotypic. These findings suggest but do not prove that a related agent, possibly the virus described by York and Baker,[12] may be responsible for the unexplained serologic findings.

The serologic status of calves in respect to antibodies against poliomyelitis virus was less complex. Five of the sixteen calves had neutralizing antibodies in serum dilutions ranging from 1:4 to 1:32 during the preinoculation period. One of the nine calves inoculated with type 2 poliomyelitis virus had an increase in serum neutralizing antibody.

* In this group are included calves 112 and 113; detailed protocols on these calves are not included in the tables, although the serologic observations are commented on and later summarized in table 6.

12. York, C. J. and Baker, J. A. 1951, A new member of the psittacosis-lymphogranuloma group of viruses that causes infection in calves. J. Exper. Med. 93: 587–604.

TABLE 6.—*Summary of observed changes in serum antibody levels after inoculation of calves with BE, and poliomyelitis or related (BE-L) viruses.*

Virus used in inoculum	Test	Virus used in test	Number of calves	Fold increase in titers			
				<4	>4, <8	>8, <16	>16
Responses observed after inoculation with specific viruses							
BE W.S.	CF	BE	11	2	1	4	4
Brunhilde (type 1)	CF	type 1	4	4			
Lansing (type 2)	CF	type 2	10	9	1		
Lansing (type 2)	N	type 2	10	9	1		
Responses observed irregardless of inoculum							
BE W.S.	CF		16	4	3	5	4
Brunhilde (type 1)	CF		16	16			
Lansing (type 2)	CF		16	15	1		
Lansing (type 2)	N		16	15	1		

CF = complement fixation.
N = neutralization.

Preinoculation serum titers of 1:32 increased 2-fold in 30 days, and 4-fold in 90 days. A concomitant rise in CF antibodies was not found. A corresponding change in serum antibodies for poliomyelitis viruses was not found in serums obtained from other experimental calves. A summary of changes observed in the serologic status of calves inoculated with poliomyelitis and BE viruses appears in table 6.

The possibility of occurrence of cross infection among calves housed in the same barn was considered at the beginning of the experiments. Four normal calves quartered in the experimental area were subjected to similar care, and studied in the same way as were calves inoculated with active viruses. None of the calves showed a rise in antibody titer against either the psittacosis-lymphogranuloma venereum group or type 2 poliomyelitis viruses. These animals were subsequently inoculated with BE viruses.

DISCUSSION

Previous observations by Chang and Wenner[3] on the serologic relationship of the virus of sporadic bovine encephalomyelitis to the Lansing strain of poliomyelitis virus are not confirmed. The virus responsible for the clinical complex referred to as sporadic bovine encephalomyelitis is a member of the psittacosis-lymphogranuloma group of infectious agents. The available evidence indicates that bovine encephalomyelitis virus does not share common antigenic components with type 2 poliomyelitis virus (Lansing). Clinical and serological data recorded in this paper provide further evidence of dissimilarities among these viruses.

The data also provide reasonable evidence that type 2 strains of poliomyelitis viruses do not cause evident or occult infection in calves. Indeed, these agents failed to provoke a specific antibody response. It does not appear probable that the occurrence of antibodies neutralizing type 2 poliomyelitis virus in serum obtained from cattle[11] is the result of poliomyelitis infection. Among the 16 calves studied, serums obtained from 5 calves neutralized Lansing virus. The serum component was present in dilutions 1:4 to 1:32 in preinoculation specimens. A change in serologic status occurred once (calf 118, a 4-fold increase) during the experimental period. It is evident that infection with BE virus did not account for the origin of the serum component capable of inactivating type 2 poliomyelitis virus.

Confirmatory evidence was obtained regarding infectivity for calves of the virus previously shown to be related to the psittacosis-lymphogranuloma venereum group. Using as a model the

naturally occurring disease, the clinical course of infection in experimental calves was much less severe than anticipated. None of the experimental calves succumbed because of encephalitis. The experimentally induced illness was quite benign, noting only the presence of fever, anorexia, and depression. Nevertheless, the serologic changes and the visceral pathologic changes observed at necropsy were similar to those observed in fatal cases occurring in affected herds.[1]

The reasons that the experimental calves did not develop encephalitis are unknown. Mild meningitis was found in three calves. The lesions were focal and quite benign compared with the insult to the central nervous system of calves succumbing during epizootics. The tests designed to enhance infectivity of BE virus with poliomyelitis viruses were not effectual.

It is our belief that infection with BE virus is widespread in distribution, that many animals experience infection, and that of the many infected, a small but unknown number develop encephalomyelitis. Severe vascular damage is undoubtedly related to the degree and duration of viremia, with subsequent change of endothelial barriers by soluble toxins. It is possible that another parasitic agent dormant in calves may be provocative in injury to the central nervous system. No evidence in support of this concept has been found in the natural disease[1,2] or in limited observations in experimental infections.

SUMMARY

The studies appearing in this report support observations reported previously that the agent belonging to the psittacosis-lymphogranuloma venereum group of viruses (BE virus) is etiologically related to the disease of cattle known as sporadic bovine encephalomyelitis.

The virus (BE-L), apparently isolated from chick embryos infected with BE viruses and related serologically to type 2 poliomyelitis viruses, failed to cause apparent or occult illness in experimental calves. Corresponding results were obtained in calves inoculated with known type 2 strains of poliomyelitis viruses. Calves inoculated with poliomyelitis viruses were not immune to challenge with BE viruses. The data indicated that poliomyelitis viruses were etiologically unrelated to the disease, sporadic bovine encephalomyelitis. The study has not confirmed an antigenic relationship among these two groups of viruses.

POLYSACCHARIDE ANTIGENS OF LEPTOSPIRES

MORRIS D. SCHNEIDER*

From the Veterinary Division, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington 12, D. C.

The object of this investigation has been to prepare, chemically characterize, and determine serologic reactivity of components possibly useful in classification of selected leptospiral serotypes, according to their immunochemical characteristics.

The complement fixation test is one of several serological reactions which has been applied to the study of leptospirosis in man and in animals. Complement-fixing antigens are crude preparations of whole cells grown in artificial mediums enriched with serum[1,2] or propagated in embryonating hens' eggs.[3] The antigens are valuable diagnostic aids due to their broad spectrum of reactivity with heterologous leptospiral antiserums,[4] but are not satisfactory for taxonomic studies.

A substance, designated fraction 1, was isolated from *Leptospira icterohemorrhagiae*.[5] This study has been extended to provide comparative information about the chemical and serological properties of analagous fractions from *Leptospira canicola* and *Leptospira bataviae*.

MATERIALS AND METHODS

Cultivation of organisms.—*Lept. icterohemorrhagiae* AB, *Lept. canicola* and *Lept. bataviae* were employed in this investigation. Their serological relationships have been studied by means of agglutination-lysis reactions.[4] Each strain was propagated for 10 days in Stuart's medium[6] enriched with 7.5% sterile, Seitz-filtered, heat inactivated, normal rabbit serum. The leptospires were harvested by centrifugation, washed twice and resuspended in isotonic salt solution containing a phosphate buffer at pH 7. The final suspension of washed cells was 2% of the culture volume. The dry weight of cells was 40 to 77 mg per liter of starting culture material. A total of 4.6 g of dry organisms was employed in this work.

Isolation of fraction 1.—Washed viable organisms were disintegrated by the addition of an equal volume of cell-disrupting fluid composed of 0.1 *M* NaCl, 0.2 *M* sodium citrate, and 0.4% sodium desoxycholate, pH 7.5 to 7.6.[5,7] One-half volume of a mixture of 4 parts chloroform and 1 part n-amyl alcohol was added.[8] The disintegrated cell preparation was shaken vigorously and stored at about 5 C. The chloroform-treated cell mass was centrifuged under refrigeration and at low speed. The product separated into (a) an upper aqueous phase, (b) an interfacial cell-protein gel, and (c) a bottom chloroform layer. Frac-

Received for publication September 30, 1953.

The starting material and hyperimmune leptospiral rabbit serums were provided by the Department of Veterinary Bacteriology. The complement fixation tests were carried out by the Department of Veterinary Clinical Pathology. The writer expresses his thanks to Dr. John F. Kent and Miss Charlotte Campbell for providing luetic serums and antiserums for protozoal and mycotic infections. To Dr. C. W. Hiatt, the author owes a special debt of gratitude for many hours of stimulating discussion and encouragement during the progress of this work.

* Present address: Veterinary Branch, Third Army Medical Laboratory, Fort McPherson, Georgia.

1. Noguchi, H. 1920, J. Exper. Med. **31**: 135–158.
2. Randall, R., Wetmore, P. W. and Warner, A. 1949, J. Lab. & Clin. Med. **34**: 1411–1415.
3. York, C. J. 1952, Am. J. Vet. Res. **13**: 117–120.
4. Gochenour, W. S., Jr. and Yager, R. H. 1952, Proc. 89th Annual Meeting A.V.M.A., 178–183.
5. Schneider, M. D. 1953, Proc. Soc. Exper. Biol. & Med. **82**: 655–659.
6. Stuart, R. D. 1946, J. Path. & Bact. **58**: 343–349.
7. McCarty, M. and Avery, O. T. 1946, J. Exper. Med. **83**: 97–104.
8. Sevag, M. G., Lackman, D. B. and Smolens, J. 1938, J. Biol. Chem. **124**: 425–436.

TABLE 1.—*Chemical composition and properties of fraction 1 antigens from leptospires.*

Analysis	*Lept. icterohemorrhagiae*			*Lept. canicola*	*Lept. bataviae*
	Preparation				
	1	2	3	4	5
Yield fraction 1, % of dry weight of cell	57	36	43	39	64
% of fraction 1					
Nitrogen	3.2	9.1	6.2	9.5	3.1
Phosphorus	1.0	1.0	1.0	1.1	0.7
Desoxypentosenucleic acid	4.0	9.1	8.9	12.4	5.2
Pentoses*	4.9	8.0	7.2	8.3	6.1
Methylpentose†	3.7	7.2	5.9	4.1	4.1
Serological activity, μg of antigen‡					
As solids	6.3	2.3	2.1	1.0	1.8
As pentoses	0.31	0.18	0.15	0.08	0.11

* Calculated as d(−)ribose.
† Calculated as l(−)rhamnose.
‡ Smallest amount of antigen which fixed complement in the presence of homologous hyperimmune rabbit serum.

tion 1 was contained in the aqueous phase. Preparations of the aqueous phase in Visking cellophane bags were dialyzed for 10 days or longer against frequent changes of 0.15 *M* NaCl solution or distilled water, at 0 C. Additional details of the procedure have been described.[5] Final volumes of fraction 1 were 4 to 6% of the original culture volume.

Analytical procedures.—Nitrogen was determined by a micro-Kjeldahl procedure employing a K_2SO_4–$CuSO_4$ digestion catalyst. Preparations were oven-dried to constant weight for 18 to 24 hours, at 95 to 105 C, for the determination of total solids. Phosphorus was estimated by a colorimetric method,[9] total pentoses according to Kerr and Seraidarian,[10] and methylpentose according to Dische and Shettles' cysteine-sulfuric acid reaction (CyR_{10}) which is heated 10 minutes at 100 C.[11] An adaptation of Dische's diphenylamine reaction was employed for the determination of desoxypentosenucleic acid.[12] Desoxypentosenucleic acid in table 1 was calculated as sodium desoxyribonucleate (Nutritional Biochemicals Corp.). Carbohydrate was detected qualitatively with Dische's naphthol reagent III.[13] A specific orcinol spray on chromatograms of hydrolysates was used for detection of ketoheptoses and ketoses.[14] Usual biuret and ninhydrin tests were applied. A sensitive alkaline copper-Folin reaction was employed for detecting trace amounts of protein.[15]

Absorption spectra.—Measurements were carried out in a Beckman Model DU Spectrophotometer. Absorption spectra were taken of reaction mixtures of orcinol-HCl-$FeCl_3$ reagent[10] with fraction 1 and a purified antigen of *Lept. icterohemorrhagiae.* The sulfuric acid-sulfhydryl (BCyR) color reaction of Dische was employed.[16]

Complement fixation tests.—All preparations of fraction 1 and purified antigens were tested against known hyperimmune rabbit serums by means of a semiquantitative complement fixation test.[17]

EXPERIMENTAL AND RESULTS

Chemical aspects

Chemical characteristics.—The dry weight and composition of washed whole leptospire cells were, respectively: *Lept. icterohemorrhagiae*, 3274 mg, total nitrogen, 7.9% (average value for three preparations); *Lept. canicola*, 590 mg, total nitrogen, 8.4%; and *Lept. bataviae*, 744 mg, total nitrogen, 6.5%. The chemical composition and properties of fraction 1 antigens from the three serotypes of leptospires are summarized in

9. Gomori, J. 1942, J. Lab. & Clin. Med., **27:** 955–960.
10. Kerr, S. E. and Seraidarian, K. 1945, J. Biol. Chem. **159:** 211–225.
11. Dische, Z. and Shettles, L. B. 1948, J. Biol. Chem. **175:** 595–603.
12. Sevag, M. G., Smolens, J. and Lackman, D. B. 1940, J. Biol. Chem. **134:** 523–529.
13. Dische, Z. 1929, Mikrochemie, **7:** 33–68.
14. Benvenue, A. and Williams, K. T. 1951, Arch. Biochem. & Biophys. **34:** 225–227.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. 1951, J. Biol. Chem. **193:** 265–275
16. Dische, Z. 1949, J. Biol. Chem. **181:** 379–392.
17. Kolmer, J. A., Spaulding, E. H. and Robinson, H. W. 1951, Approved Laboratory Technic, ed. 5, New York, Appleton-Century Croft, Inc., p. 828.

table 1. Fraction 1 comprised from 30 to 50% of the dry weight of the organism. It showed considerable chemical complexity. The preparation contained about 1% organic-bound phosphorus and from 3 to 9% organic-bound nitrogen, together with desoxypentosenucleic acid and complex polysaccharide material. Pentoses and methylpentose were among the carbohydrate residues of the polysaccharide moiety. Limited studies of chromatograms of hydrolysates of fraction 1 of *Lept. icterohemorrhagiae* have revealed the movement of 5 to 7 components. Certain of these residues migrated at rates similar to reference standards of l(−)arabinose, d(−)xylose, l(−)rhamnose and glucosamine. Fraction 1 failed to react in the biuret and ninhydrin tests. However, trace protein contaminant was detected by means of a sensitive alkaline-copper-Folin reagent. Reaction products of Dische's naphthol reagent with fraction 1 materials were strongly positive for carbohydrate.

Monosaccharide components.—Absorption spectra were determined of reaction products of orcinol-HCl-$FeCl_3$ reagent with antigens of *Lept. icterohemorrhagiae*, and standard preparations of d(−)ribose and ribonucleic acid, after heating at 100 C for 20 minutes (fig. 1). The preparations gave peaks at 430 and 670 mμ. The strong absorption band at 670 mμ is a characteristic property of pentoses. The peaks in the region of 430 mμ suggest that the pentose residues contained in fraction 1 and an "ethanol soluble" component of *Lept. icterohemorrhagiae* are probably different from d(−)ribose.

Preliminary identification of certain sugars of the polysaccharide moiety of fraction 1 has been achieved by means of a sulfuric acid-sulfhydryl color (BCyR) reaction of Dische.[16] Controls were run in the absence of cysteine under identical conditions. The following materials were examined: Fraction 1 from each of *Lept. icterohemorrhagiae*, *Lept. canicola* and *Lept. bataviae*, and fraction 1 of *Lept. icterohemorrhagiae* after hydrolysis in 1 *N* H_2SO_4 for 6 hours at 100 C, followed by neutralization with $BaCO_3$. Mixtures of pure pentose and methylpentose carbohydrates were included in the observations. The latter solutions simulated the concentrations of saccharides in fraction 1. The ultraviolet absorption spectra of typical reactive products are demonstrated in figure 2, in which curve I is, in each instance, the reaction mixture in the absence of cysteine. Curves II (primary reaction) are of sugars to which cysteine was added and measurements taken 15 to 45 minutes later. Curves III (secondary reaction) represent spectra of the same reaction mixtures after standing at room temperature for 22 to 27 hours. The spectra were measured against a reagent blank containing water in place of the sugar samples. A comparison of the different spectra of fractions 1 and the mixtures of pure carbohydrates reveals that they possess identical characteristics. The spectra of reaction mixtures plus cysteine all show maxima at or near 328 and 393 mμ, at the end of 15 to 45 minutes, and one peak located sharply at 400 mμ, at the end of 22 to 27 hours, and are characteristic of pentoses and methylpentose, respectively.

Serological aspects

Complement fixation tests.—The relative specificity of fraction 1 antigens of *Lept. icterohemorrhagiae*, *Lept. canicola*, and *Lept. bataviae* with corresponding hyperimmune rabbit serums is demonstrated in table 2. Complement fixation end points are the reciprocals of the greatest serum dilution at which a 2+ to 4+ reaction occurred. Cross reactivity is

particularly noticeable with antigens of *Lept. icterohemorrhagiae* and *Lept. canicola*. As the density of each antigen is varied, the pattern of cross reactivity undergoes change; however, at all density levels of antigen employed in the test, the active substance reacted preferentially with homologous hyperimmune serum. Since pentose appears to be a distinguishing component of fraction 1, it has served as a convenient index of the serological activity of the antigen preparation (see table 1).

"Purified" antigens of leptospires.—Partial purification of the active principle of fraction 1 has been achieved. (a) The antigen was fractionated from its aqueous salt phase (0.15 *M* NaCl) by means of three volumes of absolute ethanol. A fibrous precipitate appeared which contained virtually all of the desoxypentosenucleic acid. The active principle was in the alcoholic phase and has been designated, for convenience, the "ethanol soluble" component. Fraction 1 antigen which had been dialyzed until it was salt-free failed to give the fibrous nucleotide precipitate with ethanol. (b) After dissolving magnesium chloride (0.024 *M*) in the aqueous fraction 1, the addition of trace amounts of 10 *N* NaOH produced a heavy, flaky and sticky "alkaline insoluble" precipitate. The latter material was sedimented by centrifugation and contained a considerable quantity of the serologically active principle. (c) A departure from the previously described methods of purification was the following procedure: An aliquot of fraction 1 was mixed with one-half volume each of about 0.2 *N* H_2SO_4 and 2% sodium tungstate solution. The mixture, at pH 1.8 to 2.0, was shaken, held for 42 to 48 hours at 0 C, and centrifuged for 90 minutes at 20,000 r.p.m. The sedimented material contained the "tungstic acid precipitable" antigen.

TABLE 2.—*Complement fixation end points of fraction 1 antigens of leptospires.*

Antigen dilution	Hyperimmune rabbit serums		
	Lept. icterohemorrhagiae	*Lept. canicola*	*Lept. bataviae*
	Complement-fixation end points*		
Lept. icterohemorrhagiae			
1: 1	960*	480	20
1: 2.5	960	160	15
1: 5	640	80	15
1:10	640	80	10
1:20	480	40	1†
1:40	80	40	0‡
1:80	0	0	0
Lept. canicola			
1: 1‖	120	480	240
1:2.5	120	240	60
1: 5	120	240	60
1:10	120	240	30
1:20	30	120	15
1:40	<15¶	30	<15
1:80	<15	15	<15
Lept. bataviae			
1: 2.5	120	60	480
1: 5	15	15	480
1:10	<15	<15	480
1:20	<15	<15	480
1:40	<15	<15	240
1:80	<15	<15	30

* Figures are the reciprocals of the highest dilutions of hyperimmune leptospiral rabbit serums reacting with corresponding amount of antigen.

† 2+ to 4+ complement fixation reaction with undiluted hyperimmune serum, but complete or nearly complete hemolysis in the the next serum dilution tested (1:10).

‡ Complete or nearly complete hemolysis on testing with undiluted serum.

‖ Slightly anticomplementary at density of antigen indicated.

¶ The maximum concentration of hyperimmune serum employed in the test was a dilution of 1:15 in physiological saline.

The antigen content of each of the several preparations was determined by means of the semiquantitative complement fixation reaction. All the preparations exhibited good activity as indicated in table 3.

A preparation of "ethanol soluble" component of *Lept. icterohemorrhagiae* has been analyzed for elementary constituents.[18] The percentage composition was as follows: C, 44.88, H, 6.89, N, 6.9, P, 1.5, S, 0.18, residue, 11.43, pentoses, 19.4 and methylpentose, 14.5. Limited chemical analyses of the "alkaline insoluble" and "tungstic acid precipitable" subfractions show the presence in these preparations of 70 to 90% of the

18. Hiatt, C. W. Dec. 1952, in Symposium on the Leptospiroses, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D. C., pp. 11–12.

TABLE 3.—*Complement-fixation end points of "purified" antigens of leptospires.*

Antigen dilution	Hyperimmune rabbit serums		
	Lept. icterohemorrhagiae	*Lept. canicola*	*Lept. bataviae*
	Complement-fixation end points		
Lept. icterohemorrhagiae "75% ethanol soluble" component*			
1: 1	960†	120	20
1: 2.5	960	60	10
1: 5	960	60	1
1:10	960	30	1
1:20	240	1	1
1:40	120	1	0
1:80	30	0	0
Lept. icterohemorrhagiae "alkaline insoluble"†			
1: 1	960	160	15
1: 2.5	960	120	10
1: 5	960	30	0
1:10	480	0	0
1:20	160	0	0
1:40	30		
1:80	0		
Lept. canicola "tungstic acid precipitable"			
1: 2.5	30	120	30
1: 5	30	240	30
1:10	30	240	30
1:20	30	240	30
1:40	15	240	15
1:80	<15	120	<15
Lept. bataviae "tungstic acid precipitable"			
1: 5	60	15	480
1:10	60	<15	480
1:20	<15	<15	480
1:40	<15	<15	480
1:80	<15	<15	480
1:160	<15	<15	60

* See preparation 16[5] for serological activity of original fraction 1.
† See table 2 for explanation of figures.

TABLE 4.—*Antigens of* Lept. icterohemorrhagiae *exhibiting group reactivity in the complement fixation test.*

Hyperimmune rabbit serum†	Complement fixation end point*	
	Fraction 1	"Ethanol soluble" component
	Antigen: μg pentose(s) per ml	
	9.8	12.4
Lept. andaman	64*	64
Lept. semerang	64	16
Lept. hyos	16	4
Lept. bataviae	16	16
Lept. pomona	16	16
Lept. djasmin	16	16
Lept. sentot	16	16
Lept. autumnalis	16	16
Lept. australis A	16	16
Lept. ballum	64	16
Lept. canicola	16	16
Lept. icterohemorrhagiae	>512	>512
Lept. australis B	16	16
Lept. pyrogenes	64	64
Lept. grippotyphosa	64	64
Lept. medanensis	64	64
Lept. hebdomadis	64	64
Lept. sejro	4	4
Lept. saxkoebing	64	64
Lept. bovis Palestine	4	4
Lept. biflexa	16	16

* Reciprocal of highest dilution of antiserum reacting with antigen indicated.
† Antiserums diluted 1:4, 16, 64, 256, and 512.

starting pentoses of fraction 1.

Genus specificity of fraction 1 antigens.—The ability of fraction 1 antigens to detect group specific antibodies has been demonstrated (table 2). It was shown that changes in antigen density resulted in alteration of cross reactivity with heterologous hyperimmune serums. At approximately equivalent density of fraction 1 and "ethanol soluble" antigens, cross reactions of identical magnitude were obtained with a series of heterologous hyperimmune leptospiral rabbit serums (table 4). But the active principle reacted preferentially with the homologous hyperimmune serum.

The complement fixation reactions of fraction 1 antigens of three serotypes of leptospires is shown in table 5. The active principle was specific for the genus *Leptospira*. Fraction 1 antigens did not cross react with known positive luetic serums, and with antiserums for flagellated protozoa, malarial and mycotic agents. They reacted specifically with convalescent serums of known leptospiral infection.

Properties of the complement-fixing antigen.—The serologically reactive fraction 1 was nondialyzable through Visking cellophane tubing. The active principle was stable to heating for 1 hour at 100 C. In the aqueous phase, fraction 1 was in a finely dispersed colloidal form. Most of the serological principle failed to pass through a sintered glass bacteriological filter. The active principle was precipitable from alkaline solution containing magnesium chloride (*Lept. icterohemorrhagiae*) and from acid solution by means of 2% sodium tungstate solution (*Lept. canicola* and *Lept. bataviae*). Fraction 1 antigen was resistant to lyophilization, sonic vibration, and shaking with chloro-

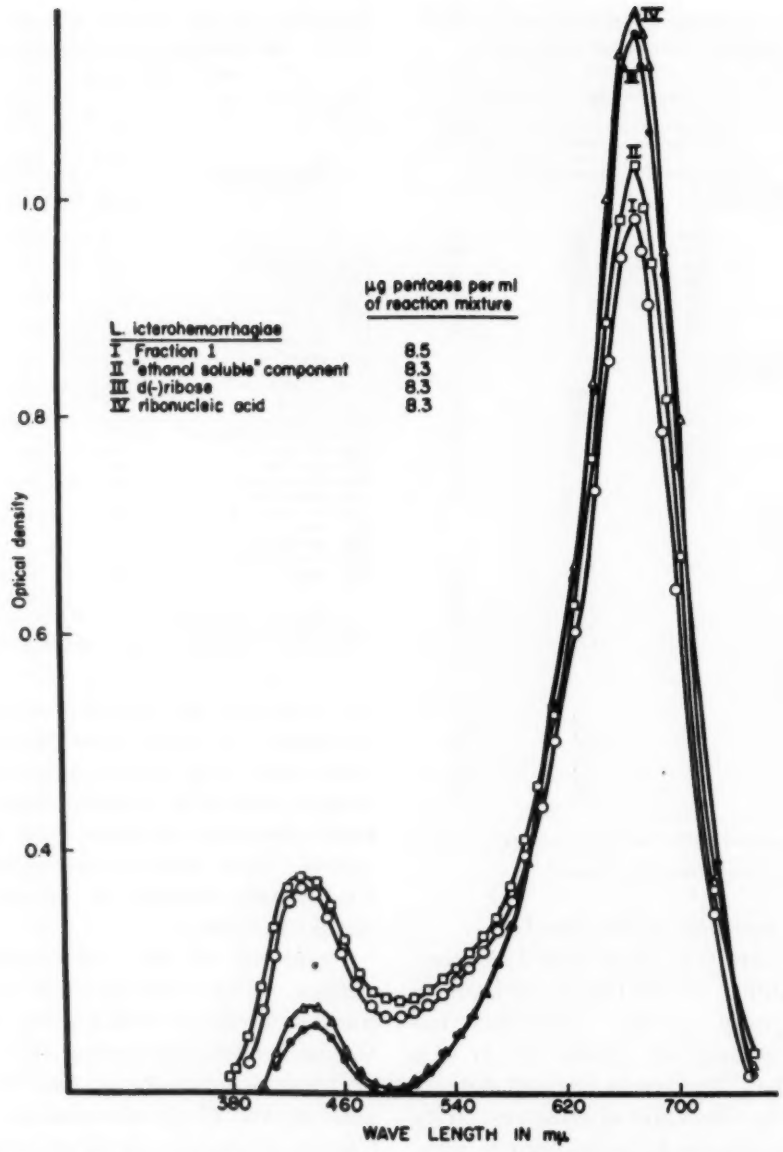


FIG. 1.—Absorption spectra of reaction products of orcinol-$FeCl_3$ reagent with antigens of *Lept. icterohemorrhagiae* and standard preparations of d(−)ribose and ribonucleic acid, after heating at 100 C for 20 minutes.

form-amyl alcohol mixture. Lyophilized preparations of fraction 1 were insoluble in hot absolute ethanol. After boiling in absolute ethanol for 1 hour, the insoluble portion of the lyophilized antigen contained virtually all of the serologically active material. After enzymatic degradation with crystalline ribo- and desoxyribonuclease (Worthington Biochemicals Laboratory, Freehold, N. J.), ultraviolet absorbing residues appeared in the dialysates of the dialyzed fractions. The polynucleotide complex of the leptospire cell isolated in fraction 1 was in this manner shown to be constituted of pentose- and

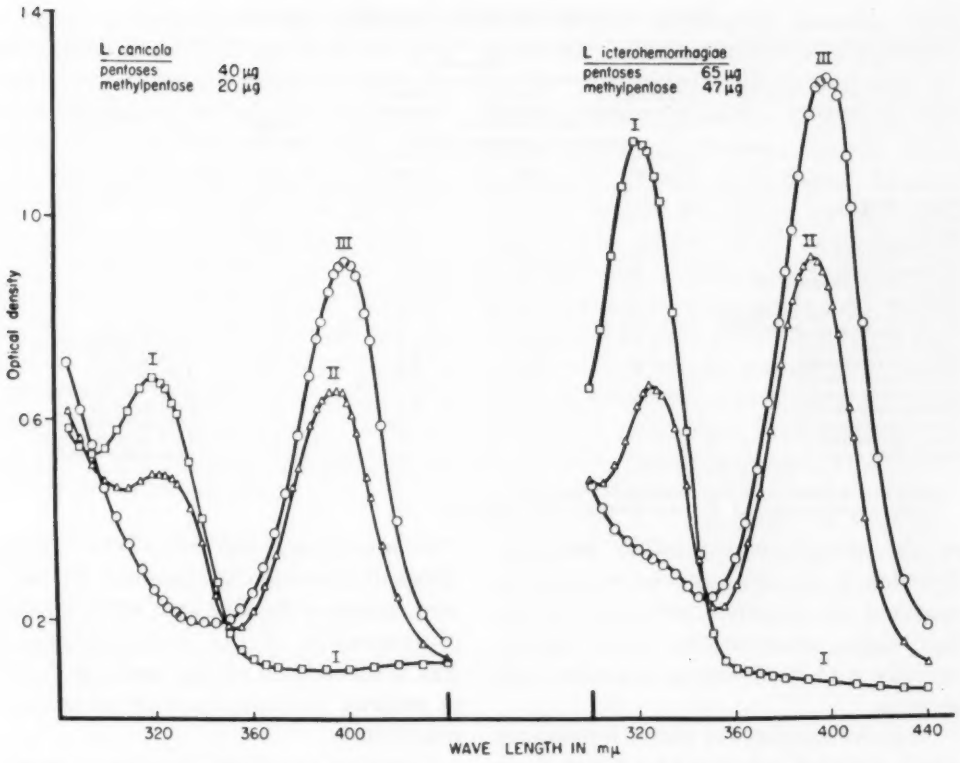


FIG. 2.—The color development of fraction 1 antigens in Dische's sulfuric acid-sulfhydryl (BCyR) reaction mixture is followed by measurements in a Beckman Model DU Spectrophotometer. The spectra of fraction 1 antigens before the addition of cysteine are given in curves I. The primary reaction products, found 15 to 45 minutes after the addition of cysteine, are shown in curves II, which are characteristic of pentoses. The secondary reaction products found 24 hours after the addition of cysteine are given in curves III, and are characteristic of methylpentoses. The amounts of pentoses and methylpentoses indicated in the antigen samples were calculated from data obtained with d(−)ribose and l(−)rhamnose.

desoxypentosenucleic acids. Since all of the active principle remained nondialyzable, the polynucleotide was virtually ruled out as the determinant of serological activity. The serologically active principle has been degraded with marked loss in complement-fixing activity, following hydrolysis for 1 hour at 100 C in (a) dilute HCl, pH 1.78 (*Lept. icterohemorrhagiae*), (b) 0.1 *N* acetic acid, pH 3.25 (*Lept. bataviae*), and (c) 1 *N* NaOH (*Lept. icterohemorrhagiae*). Fraction 1 contained organically-bound phosphorus and nitrogen; it failed to elicit an iodine-positive test but was constituted of a considerable amount of carbohydrate composed of pentose and methylpentose residues. Neither ketoheptose nor ketose was found (*Lept. icterohemorrhagiae*) by means of a specific orcinol spray test on chromatograms of hydrolysates.

DISCUSSION

The effort in this study has been directed to the isolation and identification of a cell substance common to the bodies of three serotypes of leptospires. The substance, fraction 1, from the leptospiral cell, is serologically active and possesses the property of distinguishing group specific antibodies by means

TABLE 5.—*Genus specificity of leptospiral antigens.*

Serums	Fraction 1			Serum controls	Remarks
	Lept. icterohemorrhagiae	*Lept. canicola*	*Lept. bataviae*		
	Antigen: μg pentose(s) per ml				
	10	10	10		
Human luetic	—	—	—	—	
Human luetic	—	—	—	—	
Human luetic	—	—	—	—	
Human malarial	—	—	—	—	
Human normal	—	—	—	—	
Rabbit *Trypanosoma cruzi*	—	—	—	—	
Rabbit normal	—	—	—	—	
Rabbit *Histoplasma capsulatum*	1+	—	±	—	
Rabbit *Blastomyces dermatitidis*	±	—	—	—	
Rabbit hyperimmune	4+	4+	4+	—	*Lept. icterohemorrhagiae*
Rabbit hyperimmune	4+	4+	4+	—	*Lept. canicola*
Rabbit hyperimmune	3+	4+	4+	—	*Lept. bataviae*
Human leptospiral	4+	—	—	—	*Lept. icterohemorrhagiae*
Human leptospiral	±	—	3+	—	*Lept. bataviae*
Human leptospiral *	4+	—	—	—	*Lept. icterohemorrhagiae*
Bovine leptospiral	—	—	—	—	*Lept. grippotyphosa*
Bovine leptospiral	—	—	—	—	*Lept. grippotyphosa*

2+ to 4+, positive; ± to 1+, doubtful; −, negative.
* Diagnosis of leptospirosis by means of agglutination-lysis test.

of the complement fixation reaction. Fraction 1 antigens appear to share in common an inherent antigenic factor, but they nevertheless react preferentially with homologous hyperimmune serums.

Massive numbers of viable leptospires are inoculated into the blood stream in the production of hyperimmune rabbit serums. Prepared in this manner, hyperimmune leptospiral serums are rich in group specific antibodies. The latter type antibodies are also found in the natural course of disease, but their concentration is considerably lower. Cell-free fraction 1 antigens were applied in a number of tests with a random selection of serums from known positive, naturally occurring leptospiral infections (see table 5). The complement fixation reactions were specific for the leptospire serotype, although a relatively large quantity of test antigen was employed. It is for the above reason that the application of fraction 1 antigens in specific diagnosis deserves further study.

Purification of fraction 1 antigens was undertaken primarily to illuminate the question of cross reactivity with hyperimmune serums of other serotypes. The interesting feature of the widely different attempts at chemical purification concerns the fact that while a large concentration of the active substance was achieved, all of the methods failed to restrict the spectrum of serological reactivity.

Chemical analysis has been extensively applied only to fraction 1 antigens. The chemical composition of the cell-free extracts gives some indication of the probable chemical nature of the active principle. The predominant chemical constituent of fraction 1 is a pentosan polysaccharide material. The polysaccharide and a polynucleotide moiety of the leptospire cell appear to be closely associated in this extract, but there is no evidence that they are chemically bound. The former fact, together with the knowledge that fraction 1 constitutes 30 to 50% of the dry weight of the leptospire cell, indicate that the product is largely, if not exclusively, derived from the somatic part of the leptospire cell. Analytical procedures employed in this study have not sharply differentiated the fraction 1 antigens of three serotypes of leptospires. Absorption spectra measurements indicate the presence of similar

or identical monosaccharide residues in the cell-free extracts of each of the leptospire serotypes studied. Similarities in chemical composition and the broad spectrum of serological reactions with hyperimmune rabbit serums indicate that the active principle and pentosan polysaccharide of the fraction 1 antigen are probably identical, but the exact chemical nature of the active material has not yet been fully determined. In certain respects, in chemical aspects and serological group reactivity, the active principle of fraction 1 is analogous to the somatic polysaccharide "C" of pneumococci.[19]

SUMMARY

A substance, fraction 1, has been isolated from each of *Leptospira icterohemorrhagiae*, *Leptospira canicola*, and *Leptospira bataviae*. It contains a genus-specific principle in association with a polynucleotide moiety. The antigen reacts in the complement fixation test, preferentially with homologous hyperimmune serum, but cross reacts to an appreciable extent with heterologous hyperimmune serums within the leptospire group. Nucleic acids and trace amounts of protein are probably not determinants of serological activity. Evidence is presented which suggests that the active principle is a complex polysaccharide, largely pentosan in nature, and is analogous to the somatic polysaccharide "C" substance of bacteria. Chemical differences have not yet been established for similarly prepared cell-free extracts from three serotypes of leptospires, although their serological differences are apparent.

19. Heidelberger, M. and Kendall, F. E. 1931, J. Exper. Med. **53**: 625–639.

PARTICIPATION OF THE POPLITEAL LYMPH NODE AND SPLEEN IN THE PRODUCTION OF DIPHTHERIA ANTITOXIN IN THE RABBIT

ABRAM B. STAVITSKY

From the Department of Microbiology, Western Reserve University School of Medicine, Cleveland, Ohio

The objective of this study was the development of an experimental system for the elucidation of the mechanisms of antibody formation. It was first considered worthwhile to attempt to localize the antigen and possibly, therefore, the antibody-producing "mechanism" in as few organs as feasible. Under these conditions the possibilities for the study of antibody synthesis appeared great. Restriction of antigen to a few organs might limit antigenic stimulation of the host to these organs. Residual antigen and antibody might be expected to be present most regularly and in highest concentration in these organs where initial fixation of antigen occurred. Extirpation of one or more of the organs might then remove the antigenic stimulus or the actual "mechanism" involved in antibody synthesis.

In addition to the attempt to localize the process of antibody formation, it was considered advantageous to employ the booster response to a second injection of diphtheria toxoid[1] as a phenomenon which further increased the range of experimental possibilities.

Since a great deal of evidence pointed to the participation of lymph nodes and spleen in the production of antibodies to a variety of antigens,[2–5] the role of these organs in the response to diphtheria toxoid was studied. The data presented indicate that cells derived from these organs play an important although as yet undefined role in the enhanced response to a second injection of diphtheria toxoid in the rabbit and probably in the primary response as well.

Received for publication October 7, 1953.

Preliminary reports of this work have been presented to the American Association of Immunologists at Cleveland, Ohio, in April, 1951, and at New York City in April, 1952.

The competent technical assistance of Jane Robertson, Rita Evans, Anita Jarchow, and Bradley Brownlow in various phases of this work is gratefully acknowledged.

1. Glenny, A. T. and Südmerson, J. J. 1921, J. Hyg. **20:** 176–220.

MATERIALS AND METHODS*

Antigens and antiserums.—The antigens included: (1) alum-precipitated diphtheria toxoid (DT), (2) fluid diphtheria toxoid (FT), (3) alum-precipitated tetanus toxoid (TET). Both toxoids were the "Purogenated" toxoid prepared by the procedure of Pillemer et al[6] and contained 80 Lf/ml.

The antiserums included: (1) rabbit anti-DT, (2) horse anti-DT. The rabbit anti-DT was prepared by repeated intravenous injection of 0.5 ml of alum-precipitated DT over a period of 3 weeks. The horse anti-DT contained 500 Lf/ml.

Animals.—Heterozygous albino rabbits weighing about 6 pounds were used. No segregation ac-

* The author is grateful to the Lederle Laboratories for the generous gift of large volumes of diphtheria toxoids and antitoxins.

2. Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies, ed. 2, Melbourne, MacMillan and Co.
3. McMaster, P. and Hudack, S. S. 1935, J. Exper. Med. **61:** 783–806.
4. Ehrich, W. E. and Harris, T. N. 1942, J. Exper. Med. **76:** 335–348.
5. Dougherty, T. F., Chase, J. H. and White, A. 1944, Proc. Soc. Exper. Biol. & Med. **57:** 295–298.
6. Pillemer, L., Grossberg, D. B. and Wittler, R. G. 1946, J. Immunol. **54:** 213–224.

cording to sex was made, as it did not appear to be an important factor in the study.

Blood samples.—Usually these were obtained from the central artery of the ear.

Antitoxin and toxoid determinations.—In the earlier experiments the level of antitoxin was determined by the rabbit intracutaneous method of Fraser.[7] Later it was estimated by a modification[8] of the hemagglutination reaction for antiprotein serums devised by Boyden.[9] It was found[8] that diphtheria toxoid could be adsorbed on sheep red cells which had been treated with tannic acid. These toxoid-sensitized cells were agglutinated by specific antitoxin. The sensitivity of the hemagglutination reaction was comparable to that of the in vivo reaction. At least 0.001 Lf units of rabbit antitoxin could be detected by the method.[8] Moreover, there was good correlation in the range of 0.001 to 500 units, as determined by the skin test,[7] between the concentration of antitoxin and the hemagglutination titer.[8]† No attempt was made to determine the "avidity" of the rabbit antitoxin since Jerne[11] had shown that rabbit antitoxins were usually of high "avidity." Hemagglutination was inhibited by free diphtheria toxoid and this hemagglutination-inhibition reaction[9] was used for the detection of as little as 0.001 μg of toxoid. Usually all the serums from a single experiment were titrated on a single day.

Barr[12] has reported the finding of antitoxin of poor combining quality early in the immunization of horses with diphtheria toxoid. Therefore, in several experiments a "blocking" antitoxin which combined with the toxoid-cells but did not agglutinate them was sought. The antiserum in question was mixed with the toxoid-cells and a known positive agglutinating antitoxin added. The extent of agglutination in these tubes was compared with that in tubes where normal rabbit serum was substituted for the unknown antiserum. Inhibition by the unknown serum of agglutination of the toxoid-cells by the positive antiserum would then indicate that a "blocking" type of antitoxin was present. In no instance were such "blocking" antitoxins found.

Determination of tissue nitrogen and protein.—The protein in lymphoid or splenic tissue which had been homogenized in about 0.5 ml of saline was precipitated by adding 1 ml of 20% trichloracetic acid (TCA). The TCA precipitate was separated by centrifugation. One ml of 1 *N* perchloric acid was added to the precipitate, which was then boiled in a water bath for 20 minutes. The precipitate was again separated by centrifugation. Determinations of nitrogen were made on the various fractions by a micro-Kjeldahl method.[13] Evidence of actual extraction of protein by this procedure was provided by positive Biuret tests[14] on the extracts before digestion.

Adenectomy and splenectomy.*—The surgery was conducted under ether (adenectomy) or pentobarbital (splenectomy) anesthesia and rather rigorous aseptic precautions. For adenectomy the rabbit was anesthetized and strapped down on its abdomen. The leg to be incised was extended as fully as possible with the popliteal surface upward and maintained in this position by tying the leg with leather thongs. A straight proximo-distal incision about 1 inch in length was then made in the skin of the popliteal region. Slight pressure on the lateral and medial walls of the popliteal space usually caused eversion of the soft areolo-adipose tissue in which the node was imbedded. The excess perinodal fat was removed carefully with forceps and scissors. The node itself was finally extirpated by cutting the nodal artery, vein and the efferent lymph trunk. After a search revealed no nodal remnants, the incision was closed with several cotton sutures. There were few complications from the operation. About 1% of the animals developed lymphatic fistulas which had to be drained every few days. In about 10% hematomas developed at the site of operation after the animal recovered from anesthesia and moved around in its cage. However,

† Recently, Fisher[10] also has reported the in vitro estimation of small amounts of antitoxin by means of a similar hemagglutination technique. By careful comparison with the intracutaneous method he also has established the validity of the in vitro method for this determination.

* An excellent description of the anatomy of the popliteal space and its contents is given by Furuta.[15]

7. Fraser, D. T. 1931, Tr. Roy. Soc. Canada, **25**: 175–181.
8. Stavitsky, A. B. and Jarchow, C. A. To be published.
9. Boyden, S. V. 1951, J. Exper. Med. **93**: 107–120.
10. Fisher, S. 1952, J. Hyg. **50**: 445–451.
11. Jerne, N. K. 1951, Acta path. et microbiol. Scandinav. suppl. 87.
12. Barr, M. 1951, J. Path. & Bact. **63**: 557–570.
13. Redemann, C. E. 1939, Ind. & Eng. Chem. Anal., Editions, **11**: 635–637.
14. Sutherland, E. W., Cori, C. F., Haynes, R. and Olsen, N. S. 1949, J. Biol. Chem. **180**: 825–838.
15. Furuta, W. J. 1947, Am. J. Anat. **80**: 437–506.

this did not seem to affect final healing of the wound or the antitoxic response.

The spleen was removed through an incision along the left lower costal margin. The sham operations were performed exactly as were the adenectomy and splenectomy, with the exception that no organs were removed.

Injections.—The footpad was injected through a #27 needle inserted in the fleshy ball of the foot. The direct intranodal and intrasplenic injections also were made through a #27 needle.

Preparation of tissue slices, homogenates, and cell suspensions.—Tissue slices 0.5 mm thick were made with the Stadie-Riggs tissue slicer.[16] Tissue homogenates were prepared with a glass homogenizer.[17] Cell suspensions from the lymph node were prepared by teasing the organ under liquid with teasing needles. Splenic cells were isolated by teasing the spleen with the small prongs produced at the cut edge of a 60-mesh monel wire screen. The mixture of cells and tissue debris was clarified by two filtrations through four layers of gauze. The organs were suspended in Krebs-gelatin solution[18] for teasing, and the same diluent was used for washing the cells once after gauze filtration and resuspension for injection.

IN VIVO STUDIES IN THE RABBIT

Minimum interval between primary and secondary stimulus for elicitation of secondary response.†—It was first desired to produce consistently a rapid and marked secondary antitoxic response in as short a time as possible. There were few data on this point in the literature, including the classical experiments of Glenny and Südmerson.[1] After several experiments, of which one is shown in table 1, it was decided to allow at least 3 weeks to elapse between the first and second injections of toxoid.

Effect of adenectomy on secondary response.—In the first experiment 20 Lf units of DT were injected into the left rear foot pad of rabbits. Three weeks later the left popliteal lymph nodes of half of the rabbits were removed and sham operations performed on the other rabbits. One week later all animals were inoculated with 20 Lf units in the left rear foot pad. The animals were bled at various intervals before and after the injections and surgical procedures. Table 2 illustrates the results of one of these experiments.

Removal of the node did not completely abolish the secondary response. However, there did seem to be greater antibody production in the control as compared to experimental rabbits. In all cases a definite and sizable response occurred.

These data raised the possibility that some antitoxin actually was produced in the local node. Further evidence on this point was provided by the response of rabbit 9 and 11 in table 2. There was an actual drop in circulating antitoxin in these animals during the week following adenectomy.

Further data on the occurrence of antitoxin production in organs other than the lymph node following local injection, are furnished in later experiments (tables 5 and 8).

Attempts to localize antigen in lymph node.—Since toxoid apparently was spreading beyond the local node in the previous experiments, efforts were made to localize the toxoid more completely. In the first experiment, on the assumption that 20 Lf units were too much antigen to be fixed completely by the local node, the dose of DT was decreased to 0.2 and 0.02 units in two groups of animals. In the other experiment the node was exposed surgically and the toxoid injected directly into the tissue.

---

† As used by Glenny and Südmerson,[1] the "secondary or booster response" refers to the more rapid and marked appearance of antibody which follows the second or subsequent injection of antigen compared to the slow, low level response to the first injection.

16. Stadie, W. C. and Riggs, B. C. 1944, J. Biol. Chem. **154**: 687–690.
17. Potter, V. R. and Elvehjem, C. A. 1936, J. Biol. Chem. **114**: 495–504.
18. Victor, J., Raymond, R., Valliant, J., Wagner, J. C. and Pollack, A. D. 1952, J. Exper. Med. **95**: 61–75.

TABLE 1.—*Minimum interval between two injections of diphtheria toxoid for eliciting of secondary response.*

Rabbit	Treatment	Units of antitoxin per ml serum on day:						
		7	11	14	18	21	21	28
1	40 Lf DT intravenously,	0.001	0.01	0.02	0.01			
2	days 1 and 7	0.001	0.01	0.001	0.001			
3	40 Lf DT intravenously,			0.01	0.05	0.1		
4	days 1 and 4			0.01	0.05	0.2		
5	40 Lf DT intravenously,					0.1	2.0	15.0
6	days 1 and 21					0.1	5.0	15.0

Ten to twelve hours later 50 units of rabbit antitoxin were injected intravenously to combine with any toxoid that might have escaped from the local node. It was hoped that the combination of alum to fix the toxoid locally and the systemically injected antitoxin would result in essentially local antitoxin formation.

The first experiment did not reveal any definite effect of removal of the node on the secondary response to 0.2 and 0.02 units of toxoid. With these concentrations of antigen very small amounts of antitoxin were produced in the sham-operated animals. It was, therefore, impossible to distinguish clearly between the levels of antitoxin in these compared to adenectomized animals.

The second experiment yielded clear-cut data. Some of these are summarized in table 3. They show a great difference in antitoxin production in the sham-operated animals and those from which the node was extirpated. Better localization of antigen apparently was achieved in this experiment. However, complete restriction of antigen did not occur. This was evident from the results of a tertiary injection of toxoid into rabbit 15, which was previously adenectomized. The definite response which ensued (table 3) suggests that the toxoid had gotten beyond the local node. When the node was removed several days after the second injection of toxoid in other rabbits the antitoxin level continued to rise.

Again several animals, like #14 and 15, in the experimental group showed a definite drop in antitoxin during the week after removal of the node and before the second injection of toxoid.

Effect of splenectomy on secondary response.—Since the spleen also has been shown to participate in antibody production[2] its role in the secondary response was investigated. The approach was similar to that with the lymph node, with the exception that the toxoid was injected either intravenously or directly into the spleen. The details of procedure and typical results are shown in table 4.

The data definitely implicate the

TABLE 2.—*Effect of adenectomy on secondary response to locally injected diphtheria toxoid.*

Group	Rabbit	Units of antotoxin per ml serum on day:								
		1	22	30	37	45	84	136	161	169
Control	7	0.001	0.1	0.1	*18.0*	12.0	3.0	0.5	0.02	4.0
	8	0.001	0.2	0.2	*14.0*	3.0	2.0	0.2	0.02	3.5
Experimental	9	0.001	0.2	0.05	*6.5*	2.0	0.2	0.2	0.01	6.5
	10	0.001	0.1	0.3	*4.0*	2.0	0.2	0.1	0.02	8.5
	11	0.001	0.1	0.05	*6.5*	1.5	1.0	0.2	0.02	4.5

Control: day 1, 20 Lf DT; day 22, sham adenectomy; day 30, 20 Lf DT; day 161, 20 Lf DT.
Experimental: day 1, 20 Lf DT; day 22, adenectomy; day 30, 20 Lf DT; day 161, 20 Lf DT.

TABLE 3.—*Effect of adenectomy on secondary response to toxoid injected into local lymph node.*

Group	Rabbit	Units of antitoxin per ml serum on day:									
		1	21	28	36	41	46	54	76	79	83
Control	12	0.001	0.04	0.1	3.0	3.0	1.5	0.5			
	13	0.001	0.04	0.1	3.0		1.0				
Experimental	14	0.001	0.04	0.02	0.3		0.1	0.01			
	15	0.001	0.04	0.02	0.25		0.1	0.1	0.1	0.75	1.5

Control: day 1, 0.2 Lf DT; day 21, sham adenectomy; day 28, 2 Lf DT.
Experimental: day 1, 0.2 Lf DT; day 21, adenectomy; day 28, 2 Lf DT.
Rabbit 15: day 76, 2 Lf DT.

spleen in the secondary response, particularly that which followed the direct intrasplenic injection of toxoid.

Relative importance of local lymph node and spleen in secondary response to local and intravenous toxoid.—The relative importance of the lymph node and spleen in the process was studied next. Toxoid was injected either into the footpad or intravenously. At various intervals thereafter the spleen and/or lymph node were removed and within 7 to 14 days the second injection of toxoid was given. The results of one of these experiments are shown in table 5. In agreement with previous experiments (tables 2, 3, and 4), following local injection, adenectomy, and after intravenous injection, splenectomy resulted in a diminished secondary response. Splenectomy caused a lowered response even when antigen was inoculated into the foot pad. On the other hand, removal of the local node had little effect on the response following intravenous toxoid. Under the conditions of the experiment the spleen and popliteal node were involved in the secondary response following local antigen, whereas the spleen and not the local node were connected with the response to intravenous antigen. Thus the spleen was an important factor in the response regardless of the route of administration of antigen.

The effect of adenectomy and splenectomy on the level of antitoxin before the second injection of toxoid on day 34 is striking. In every instance the adenectomized and splenectomized rabbits showed a fraction of the antitoxin present in the sham-operated animals. These data implicate these organs in the primary as well as secondary response to toxoid.

*Passive transfer of lymph node and splenic cells from immunized to normal rabbits.**—The preceding experiments

* Simultaneous with the preliminary report of the author on the successful cellular transfer with appearance of antitoxin in the recipients, Wager and Chase[19] reported similar observations before the American Association of Immunologists, April 17, 1952.

TABLE 4.—*Effect of splenectomy on secondary response to toxoid injected into spleen or intravenously.*

Group	Rabbit	Units of antitoxin per ml serum on day:					
		1	21	28	36	41	46
Control	16	0.001	0.01	0.1	12.0	5.0	5.0
	17	0.001	0.02	0.2	14.0	8.0	6.0
	18	0.001	0.02	0.2	18.0	12.0	10.0
Experimental	19	0.001	0.02	0.02	0.5	0.2	0.2
	20	0.001	0.02	0.01	1.0	0.4	0.2
	21	0.001	0.02	0.02	1.0	0.2	0.2
	22	0.001	0.02	0.02	3.0	0.2	0.2

Control: rabbit 16, day 1, 2 Lf DT intravenously; day 21, sham splenectomy; day 28, 2 Lf DT intravenously.
Control: rabbits 17 and 18, day 1, 2 Lf DT into spleen; 10 hours later, 50 units rabbit antitoxin intravenously; day 21, sham splenectomy; day 28, 2 Lf DT intravenously.
Experimental: rabbits 19 and 20, same as 16 but splenectomy performed.
Experimental: rabbits 21 and 22, same as 17 and 18 but splenectomy performed.

TABLE 5.—*Relative importance of popliteal lymph node and spleen in secondary antitoxic response to locally and intravenously injected toxoid.*

Rabbit	Treatment	Reciprocal hemagglutination titer serum on day:					
		1	34	37	41	44	48
23	40 Lf DT footpad days 1 and 34; sham adenectomy and splenectomy day 27	20	2560	10,000	40,000	40,000	20,000
24		0	5120	10,000	20,000	40,000	20,000
25	40 Lf DT intraven. days 1 and 34; sham adenectomy and splenectomy day 27	0	2560	10,000	40,000	40,000	40 000
26		0	5120	10,000	40,000	40,000	20,000
27	40 Lf DT footpad days 1 and 34; adenectomy day 27	0	160	320	640	320	640
28		0	160	80	160	80	320
29	40 Lf DT footpad days 1 and 34; splenectomy day 27	0	80	320	640	320	640
30		0	160	320	640	640	320
31	40 Lf DT intraven. days 1 and 34; splenectomy day 27	0	0	40	640	106	80
32		0	0	40	1,280	1,280	320
33	40 Lf DT intraven. days 1 and 34; adenectomy day 27	0	20	320	10,000	10,000	10,000
34		0	20	640	10,000	20,000	20,000
35	40 Lf DT intraven. days 1 and 34; adenectomy and splenectomy day 27	0	0	40	640	320	80
36		0	0	80	1,280	640	640

had shown that the lymph node and spleen participated in the secondary response to diphtheria toxoid. They did not, however, indicate how closely involved in the process these organs were. The next series of experiments was directed to this aspect of the problem. Since Chase[20] and the Harrises[21] had observed the appearance of antibody in rabbits injected with lymphoid and splenic cells from immunized rabbits, their methods were used.

In many instances, as is apparent from tables 6, 7, and 8, the transfer of lymphoid and splenic cells resulted in the appearance of measurable though small amounts of specific antitoxin in the recipients. The specificity of this antitoxin was proven by the ability of diphtheria toxin or toxoid to inhibit the agglutination of toxoid-red cells by these serums derived from the recipient animals. In a few experiments, like the experience of Chase[20] with other antigens, antitoxin did not appear in the

19. Wager, O. A. and Chase, M. W. 1952, Fed. Proc. 11: 485.
20. Chase, M. W. 1951, Fed. Proc. 10: 404.
21. Harris, S. and Harris, T. N. 1951, Fed. Proc. 10: 409.

TABLE 6.—*Appearance of antitoxin in recipient following transfer of splenic cells from immunized rabbits.*

Rabbit	Day	Treatment	Units antitoxin per ml serum
37	1	16 Lf DT intravenously	0.001
	40	80 Lf DT intravenously	0.001
	47	Splenic cells transferred to 38 intravenously	25.0
38	1	Received splenic cells from 37	0.01
	2	Bled	0.01
	3	Bled	0.01–0.02
	4	Bled	0.02–0.05
	6	Bled	0.5
	9	Bled	0.05
	31	32 Lf DT intravenously	0.01
	35	Bled	0.01
	38	Bled	0.01
	41	Bled	0.01

TABLE 7.—*Appearance of antitoxin in recipient following transfer of lymph node cells from immunized rabbits.*

Rabbit	Day	Treatment	Units antitoxin per ml serum
39	1	20 Lf DT footpad	0.001
	21	40 Lf DT footpad	0.1
	28	Lymph node cells transferred to 40 intravenously	20
40	1	Received lymph node cells from 39	0.001
	3	Bled	0.01
	5	Bled	0.01–0.02
	7	Bled	0.1
	9	Bled	0.08
	11	Bled	0.05
	20	Bled	0.01

TABLE 8.—*Frequency of successful cell transfers under various experimental conditions.*

Group No.	Treatment of donors	Type cells transferred	Number of recipients	Number showing positive transfer*
1	16 Lf Dt intraven. day 1 80 Lf DT intraven. day 40 Day 47 cells transferred	Splenic	7	5
2	Same as 1	Lymphoid†	7	0
3	20 Lf DT footpad day 1 40 Lf DT footpad day 40 Day 48 cells transferred	Lymphoid	7	3
4	Same as 3	Splenic	7	2
5	Same as 3 but 21 days between injections	Lymphoid	7	1

* A transfer was considered positive when the recipient animal attained at least 0.1 unit of antitoxin or a 1:40 hemagglutination titer within 7 days after cell transfer. In no case did the recipients' serums contain more than 0.001 unit of antitoxin or show more than a 1:5 to 1:10 titer before transfer.
† Derived from lymph node.

recipients when the donor cells were held at 5 C overnight or frozen and thawed six times before transfer.

Table 8 presents data on the frequency of successful transfers under various experimental conditions. It is again to be noted that the spleen participates in the process regardless of the route of injection of antigen, whereas the lymph node is involved mainly following local inoculation. Thus these data substantiate those previously presented in table 5.

Rigorous efforts were made to exclude the transfer of toxoid or antitoxin as the basis of the positive antibody transfer. The transfer of toxoid seemed an unlikely explanation because the primary response to diphtheria toxoid usually is low and delayed for many weeks, whereas the antitoxin appeared in the recipient rabbits in 5 to 7 days. Nevertheless, these two possibilities were tested in several ways.··

Response of recipient rabbits to the injection of toxoid.—If appreciable toxoid had been transferred to the recipient with the cells, it would be expected that the injection of toxoid several weeks later would cause a typical secondary response. However, only once in 13 trials in recipients which showed a successful transfer did such a secondary response occur (see rabbit 38 in table 6). Moreover, in only three of the recipients in which antitoxin appeared 4 to 5 days after cell transfer was there observed another peak of antitoxin production some 10 to 12 days later. These results were in marked contrast to those of Wager and Chase.[19] These investigators gave larger amounts of toxoid to the donors and noted a delayed appearance of antitoxin in the recipient, following the primary antitoxic peak due to the transfer. The delayed antitoxic reaction apparently was part of the primary response to toxoid carried with the cells into the recipients. It is probable that the differences between our results and those of Wager and Chase[19] were due to the larger amounts of toxoid they employed. The higher percentage of successful cell transfers observed by Wager and Chase[19] is also in line with their use of more toxoid in the donors, since the success of the transfer probably depends on the extent of antibody production in the donor.

Presence of toxoid in lymph node and spleen cells from immunized rabbits.—The previous data had indicated that usually the donor cells did not contain enough antigen to primarily sensitize the recipients to injection of toxoid. However, it was not known whether these cells contained enough antigen to

TABLE 9.—*Presence of toxoid in rabbit tissues shown by elicitation of secondary response in primarily immunized mice.*

Mouse	Group	Treatment of mice	Reciprocal hemagglutination titer on day:	
			22	29
1	Control	Day 1—8Lf DT subcut.	0	0
2		Day 22—0.01 μg DT	0	0
3		Subcut.	0	0
4	Control	Day 1—8Lf DT subcut.	0	604
5		Day 22—0.1 μg DT	0	320
6		Subcut.	0	640
7			0	5,120
8	Control	Day 1—8Lf DT subcut.	0	10,240
9		Day 22—1.0 μg DT	0	10,240
10		Subcut.	0	10,240
11	Experimental	Day 1—8Lf DT subcut.	0	0
12		On day 22 rabbit	0	320
13		Splenic cells	0	640
14		Subcut.	0	2,560

#11, spleen from nonimmunized rabbit; #12, 13, 14, spleens from rabbits given 40 Lf DT on day 1 and spleen removed on day 22.

produce a secondary antitoxic response in a primarily sensitized animal. This was tested in mice and rabbits. Representative data are shown in tables 9 and 10.

The results indicate that in every instance the transferred cells contained enough antigen to produce a secondary antitoxic response in primarily sensitized mice and rabbits. However, despite these in vivo results it was not possible to detect toxoid in the cells by hemagglutination-inhibition employing various types of tissue and cell extracts. In no instance would these extracts inhibit hemagglutination of DT-sensitized red cells by antitoxin, although toxoid-specific material could be found by this method in the serum from rabbits injected with toxoid several days previously.

TABLE 10.—*Persistence of diphtheria toxoid in local lymph node.*

Rabbit	Treatment	Units of antitoxin per ml serum:		
		1	30	37 days
41	20 Lf DT into footpad; lymph node removed 3 weeks later, cells to #42			
42	Day 1—16 Lf DT intraven.; day 30–34 lymph node cells intraven., from #41	0.02	0.25	2

Presence of antitoxin in donor cells.—Many attempts were made to demonstrate antitoxin in association with donor cells by the intracutaneous[7] or hemagglutination methods.[8,9] The most antitoxin ever detected by these exceedingly sensitive methods was 0.25 unit in the cells from a spleen or lymph node. It is possible that this 0.25 unit was due to contamination of the cells with plasma which usually contained at least 10 units of antitoxin per ml or a titer of about 1:40,000. Further evidence against the antitoxin in the recipient merely being passively transferred with the donor cells was furnished by the lag of 4 to 5 days between the transfer and the appearance of antitoxin. Moreover, in at least one experiment (table 11) it was found that there was more antitoxin protein in the serum of the recipient at the peak of the response than total protein present in the splenic cells which were transferred. As shown in the same table, this was not usually the case. However, it is not known what percentage of the total protein in the donor cells was toxoid protein, γ globulin and hemoglobin. The total amount of antitoxin protein appearing within the recipient was not determined. It is possible that were this calculation made

TABLE 11.—*Toxoid, antitoxin and total protein content of tissues and cells from donors and antitoxin protein content of serum from recipients.*

Rabbit	Treatment	Preparation analyzed	Mg total protein	Mg antitoxin protein*
43	Day 1–16 Lf DT intraven.; day 40–80 Lf DT intraven.	Splenic cell homogenate from day 47	0.1	
44	Same as rabbit 43	Splenic tissue homogenate day 47	0.22	
45	Same as rabbit 43	Splenic cell homogenate day 47	0.34	
46	Received splenic cells from rabbits treated as were rabbits 43–45	Serum		0.15
47	Same as rabbit 46	Serum		0.075
48	Same as rabbit 46	Serum		0.049

* Calculated on basis of units of antitoxin present in recipient's serums at height of response to transferred cells and according to data of Cohn and Pappenheimer.[22] 2.4 μg N/unit antitoxin × 6.25 = 15 μg protein/unit.

it would be found that much more antibody protein consistently appeared in the recipient than the total protein transferred with the cells.

A few attempts were made to detect "blocking" antitoxin in the transferred cells. All attempts were unsuccessful.

IN VITRO STUDY OF LYMPH NODE AND SPLENIC SLICES AND CELLS FROM IMMUNIZED RABBITS

Since the previous experiments indicated that lymph node and splenic cells participated in the secondary response, in vitro methods were employed for the elucidation of their role in the process. Tissue slices from the lymph node and spleen of immunized rabbits were suspended in a rabbit serum-phosphate medium previously used in similar studies[23] and their oxygen uptake measured in the Warburg apparatus[24] for 5 to 6 hours under an atmosphere of 5% CO_2–95% O_2. Since a good rate of oxygen consumption was observed during the period of the experiment this medium was employed for the suspension of tissue slices and cells in the following experiments in the Dubnoff apparatus.[25]

In vitro release of diphtheria antitoxin from tissues of immunized animals.—Rabbits were given two injections of DT, separated by an interval of 3 weeks. At various times after the second injection the animals were bled from the heart as completely as possible and slices prepared of their organs. About 200 mg of slices were suspended in 2 ml of the serum-phosphate medium[23] in a 20-ml beaker and shaken for 5 hours at 37 C under 5% CO_2–95% O_2 in the Dubnoff apparatus.[24] The contents of the beakers were removed and the cells separated from the medium by centrifugation. Antitoxin determinations were made on the tissue and medium before and after incubation.

The data from two rabbits in one of the experiments are shown in table 12. They suggest that the antitoxin which appeared in the medium after shaking of the slices was associated originally with the slices. There was little evidence of net synthesis of antitoxin in vitro, although occasionally (rabbit 37, spleen) more antitoxin was found in the beaker at the end of the shaking period than was present in the beginning. However, in this same rabbit an apparent increase in antitoxin was shown by the kidney,

22. Cohn, M. and Pappenheimer, A. M., Jr. 1949, J. Immunol. **63**: 291–312.
23. Roberts, S., Adams, E. and White, A. 1949, J. Immunol. **62**: 155–170.
24. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. 1949, Manometric Techniques and Tissue Metabolism, ed. 2, Minneapolis, Burgess Publishing Co.
25. Dubnoff, J. W. 1948, Arch. Biochem. **17**: 327–336.

TABLE 12.—*In vitro release of diphtheria antitoxin from tissues of immunized rabbits.*

Rabbit	Tissue	Titer of tissue before incubation	Titer of tissue after incubation	Titer of medium after incubation
49	Right node	2560	160	1280
	Left node	640	160	640
	Lung	320	160	320
	Liver	320	80	320
	Spleen	320	0	320
50	Right node	640	160	640
	Lung	320	0	320
	Liver	320	0	160
	Spleen	0	0	640
	Kidney	320	0	2560

Rabbit 49, 20 Lf DT left footpad day 1; 20 Lf DT left and right footpads day 30; tissues removed day 36.
Rabbit 50, 20 Lf DT left footpad day 1; left adenectomy day 22; 20 Lf DT left and right footpads day 30: tissues removed day 36.

an organ which is probably not concerned with the production of antibody.[23] There was little correlation between the amount of antitoxin found in a particular tissue and the route of injection of toxoid as would be expected if the phenomena in vitro were related to the actual sites of antitoxin formation in vivo. The antitoxin may have been adsorbed on the tissue from the surrounding plasma, which had a titer of 1:40,000 when the tissues were removed. Therefore, these data probably reflect release of protein from the tissue slices rather than actual synthesis of antitoxin.

Study of cell suspensions in vitro.—When similar experiments were performed with isolated cells from the organs of immunized rabbits the results were inconsistent. In general, they were similar to those with the slices, with the exception that there was much less antitoxin associated with the cells before incubation than with the slices. These findings were consistent with the adsorption of antitoxin by the slices and the removal of much of this protein in the process of preparing the washed, isolated cells. They were also consistent with previous unsuccessful attempts in this laboratory and elsewhere[20,21] to demonstrate "preformed" antibody in cells from immunized animals. Because of the low level of antitoxin in the cells, it was not possible to determine whether they effected a net synthesis of antitoxin in vitro.

Attempts to produce diphtheria antitoxin by "culture" of cells and slices in vitro.—Thus far all attempts to obtain evidence of synthesis of antitoxin by slices or cells from immunized rabbits in various types of mediums in roller-tube cultures or under perforated cellophane in Stender dishes have been negative. Lymph node and spleen slices and cells from toxoid-immunized rabbits have been suspended in Earle's medium[26] or a modification* for periods ranging from 12 to 144 hours, and the tissues and medium tested at various intervals for diphtheria antitoxin by the hemagglutination method. Only release of antitoxin from the tissues has been observed.

DISCUSSION

Under the experimental conditions described, the popliteal lymph node and spleen were definitely involved in the enhanced response to a second injection of toxoid in the rabbit. Failure of Burnet[2] to observe formation of antitoxin in the local lymph node of the rabbit following local injection of staphylococcus toxoid was probably due to use of a soluble form of the toxoid which was not appreciably localized in the local node. Oakley et al[27] have presented evidence of different type from the present study for the participation of the regional nodes in antitoxin pro-

* 40% fresh, sterile rabbit serum, 40% Earle's salt solution,[26] and 20% splenic extract (1 rabbit spleen extracted with 1 cc of rabbit serum and 1 cc of saline).

26. Earle, W. R. 1948, Proc. Soc. Exper. Biol. & Med. **67**: 40–46.
27. Oakley, C. L., Warrack, G. H. and Batty, I. 1949, J. Path. & Bact. **61**: 179–194.

duction, following subcutaneous booster injections of alum-precipitated diphtheria and tetanus toxoid into guinea pigs, rabbits and horses. Also, since the completion of the present work, Freund and coworkers[28] and Coons et al[29] have presented data suggesting local production of diphtheria antitoxin.

Although the lymph node and spleen were shown to participate in the booster response, other data clearly showed that the process also involved other parts of the body. When, however, the antigenic stimulus was largely confined to the lymph node or spleen, removal of these organs virtually abolished the antitoxic response. These data are compatible with recent experiments by Oakley et al,[30] which indicated that the booster response to diphtheria toxoid might involve many sites, depending on the route of the booster injection.

The combined adenectomy and splenectomy experiments connected the spleen with the process no matter where toxoid was injected, whereas the node functioned only following local injection. The situation was only partly analogous to that observed with other antigens and test animals[2,23] where the spleen was instrumental in antibody formation when antigen was inoculated intravenously and the node after local inoculation. However, recent data obtained by Thorbecke and Keuning[31] also indicate that the spleen produces antibody following local as well as systemic injection of antigen.

Osterlind[32] observed that after primary injection of diphtheria toxin into rabbits the maximal histologic reaction occurred in the local lymph node. Following a second local inoculation the same node again responded. In the present experiments there was evidence that the node and spleen were involved in both the primary and secondary antitoxic responses. However, these observations do not help to elucidate the mechanism of the booster response. According to one concept[33] the secondary response represents release of stored "preformed antitoxin." Yet even with extremely sensitive methods only small amounts of antitoxin were detected in tissue extracts, slices and cells of immunized rabbits in this study, and contamination of these preparations with plasma antitoxin could not be excluded. The appearance of antitoxin only after a period of several days rather than immediately following cell transfer also indicated that relatively little antitoxin was associated with the cells. Further study is required to determined the relationship between the primary and secondary responses and whether the booster response represents release of "preformed antitoxin" or an enhanced rate of a synthesis which is in progress.

The mechanism by which extirpation of the node and spleen before the booster injection reduces the secondary response is not known. Removal of antigen with these organs naturally first comes to mind. However, this does not account for all of the facts. When extracts of the extirpated organs were injected into normal rabbits little antitoxin was produced. Subinoculation of these extracts into primarily immunized

28. Freund, J., Schryver, E. M., McGuiness, M. B. and Geitner, M. B. 1952, Proc. Soc. Exper. Biol. & Med. **81**: 657–658.
29. Coons, A. H., Leduc, E. H. and Connolly, J. M. 1933, Fed. Proc. **12**: 439.
30. Oakley, C. L., Batty, I. and Warrack, G. H. 1951, J. Path. & Bact. **63**: 33–34.
31. Thorbecke, G. J. and Keuning, F. J. 1953, J. Immunol. **70**: 129–134.
32. Osterlind, G. 1938, Acta path. et. microbiol. Scandinav., suppl. 34.
33. Holt, L. B. 1950, Brit. J. Exper. Path. **31**: 233–241.

rabbits and mice did produce a booster response (tables 9 and 10), suggesting that some toxoid was present. Yet at the time of adenectomy or splenectomy it could be determined indirectly that about 10 to 20% of the originally injected toxoid was present in the lymph node or spleen.[34] Therefore, it has been assumed that adenectomy and splenectomy were effective because of removal of an integral part of the system which actually synthesizes antitoxin.

The transfer experiments with isolated lymphoid and splenic cells suggest that these cells participate in antitoxin formation. The transfer of antitoxin or toxoid per se seems to have been excluded rather definitely as the basis of the transfer phenomenon. Harris and Harris,[21] Wager and Chase,[19] Wesslen,[35] and the present study were unable to demonstrate enough antibody in the transferred cells to account for the amount of antibody which appeared in the recipients. The transfer of a metabolic derivative of antigen, as postulated by Topley,[36] or some other type of intermediate in the formation of antitoxin cannot be excluded by the present data. However, Chase[37] has shown recently that the avidity of the antitoxin arising in the recipient animals reflects the quality of the antitoxin being produced by the respective donors at the time of sacrifice. Chase[37] believes that these data make it improbable that antitoxin arises in the recipient because of the transfer of an "intermediate" type of antigen.[36] Further quantitative studies are required to determine whether there is enough protein in the transferred cells to account for a type of "bound" or "blocking" antitoxin or serologically inactive precursor of antitoxin which could not be detected by the hemagglutination reaction.

It is impossible to understand the basic mechanisms of the cell transfer from the available data. The process whereby antitoxin appears in the recipients might be due either to the survival in the recipient of the transferred cells or to appropriation and utilization by the host cells of those elements of the transferred cells essential to the process. The fate of the transferred cells in the recipient is unknown, although by analogy with the general disposition of foreign particles and one pertinent study[38] they probably would be rapidly phagocytosed and broken down in the reticulo-endothelial system.

Thus far antitoxin production has not been observed when cells from immunized rabbits were placed in various mediums in vitro. This is not altogether surprising since about 5 days were required before the antitoxin appeared in the blood of recipients of cells from immunized animals. The "culture" of predominantly round cells of the lymphoid type probably did not proliferate but only survived in vitro. However, the inability to produce antibody with tissue slices incubated in vitro seems to be at variance with recent reports of antibody production by tissue fragments under similar circumstances.[31,39] Without suitable evidence many reasons can be offered for our failure, such as use of inadequately immunized donors, removal of cells from donors at incorrect time for maximal in vitro activity, unsuitable medium or techniques for in vitro antitoxin production. These factors are be-

34. Stavitsky, A. B. Unpublished experiments.
35. Wesslen, T. 1952, Acta dermat.-venereol. **32**: 265–270.
36. Topley, W. W. 1930, J. Path. & Bact. **33**: 339–351.
37. Chase, M. W. 1953, Fed. Proc. **12**: 438.
38. Farr, R. S. 1951, Anat. Rec. **109**: 515–534.
39. Fagraeus, A. 1948, J. Immunol. **58**: 1–14.

ing considered in our present studies along these lines.

It is hoped that intensive study of the process of antitoxin production by means of various modifications and permutations of the extirpation, cell transfer and in vitro experiments will yield more information on the mechanisms of this synthesis, which may also be pertinent to the more general problems of antibody and protein synthesis.

SUMMARY

The popliteal lymph node and spleen were implicated in the enhanced antitoxic response to a second injection of diphtheria toxoid in the rabbit. Following local injection of antigen, adenectomy and splenectomy reduced the magnitude of the response to a second injection, whereas after intravenous antigenic stimulation only splenectomy reduced the secondary response. Further evidence of the involvement of lymph node and splenic cells in antitoxin formation was provided by cell transfer experiments. After local injections of antigen both lymph node and splenic cells could be used for passive transfer of antitoxin, whereas after intravenous injection only splenic cells were suitable. Possible mechanisms for the participation of these cells in antitoxin production were discussed. Thus far attempts to demonstrate synthesis of diphtheria antitoxin by cells or slices of immunized animals in vitro have been unsuccessful.

MAY-JUNE 1954 NUMBER 3

The Journal of Infectious Diseases

TABLE OF CONTENTS

PAGE

S. Oshima and D. Schlessinger. The use of streptomycin-dependent and -resistant strains for the differentiation of the immunizing effect of living *Brucella abortus* vaccine in white mice 213

Arthur Zuckerman and Irwin Yoeli. Age and sex as factors influencing *Plasmodium berghei* infections in intact and splenectomized rats 225

[illegible]. The course of infection with *Plasmodium berghei* in inbred [illegible] mice 233

[illegible]. The cellular reactions to infections with *Plasmodium berghei* in the white mouse 241

Ruth E. Lasser, Jr. and George J. Race. A histopathologic study of the upper small intestine of immunized and nonimmunized mice infected with *Trichinella spiralis* 261

M. Evans, Velma C. Cheatham, Wayne M. Smith and Pamela H. Dykes. Growth of encephalitis viruses in extraneural tissues. V. Survey of susceptibility of various animal species to capacity to support multiplication of mouse neurotropic virus in vitro 271

[illegible] W. Wilcox, Robert [illegible] Meagher, and Gerald S. [illegible]. Sporadic bovine encephalomyelitis. I. Reproduction of the disease with particular reference to the virus of poliomyelitis virus in experimental [illegible] [illegible] in calves 279

[illegible] D. Schlesinger. Passive protective antibody of leptospirosis 289

[illegible] B. Stavitsky. Participation of the popliteal lymph nodes and spleen in the production of diphtheria antitoxin in the rabbit 297